ANNALES MEDICINAE EXPERIMENTALIS ET BIOLOGIAE FENNIAE

VOL. 30 1952 FASC. 1

MERCATORIN KIRJAPAINO
HELSINKI, FINLAND

Annales Medicinae Experimentalis et Biologiae Fenniae

is a direct continuation of the *Acta Societatis Medicorum Fennicae ›Duodecim‹*, 1919—1930 (Vols. I—XII) and the *Acta Societatis Medicorum Fennicae ›Duodecim‹, Ser. A*, 1931—1946 (Vols. XIII—XXIV).

The journal is published by the Finnish Medical Society ›Duodecim‹ with the object of providing an opportunity to publish articles on experimental medicine and on related biological subjects.

Each number of the journal contains 80—100 pages, three numbers forming one volume. Articles are accepted for publication with the understanding that they are original contributions never previously published. The manuscripts should be in English, French or German, and typewritten. They should not exceed a total length of 24 pages, and there should be a short summary at the end of the article.

The subscription price is Fmks 750 per volume in Finland and $ 4 or Swedish Crowns 15.00 in foreign countries. More extensive works, published as supplements the subscribers receive free of charge.

Address for subscription, exchange of reviews, and all communications:

Annales Medicinae Experimentalis et Biologiae Fenniae

Yrjönkatu 17, Helsinki, Finland.

Annales Medicinae Experimentalis et Biologiae Fenniae

est une suite directe des revues *Acta Societatis Medicorum Fennicae ›Duodecim‹* 1919—1930 (V. I—XII) et *Acta Societatis Medicorum Fennicae ›Duodecim‹* Ser. A, 1931—1946 (V. XIII—XXIV).

La revue est éditée par la Société de médecins finnois ›Duodecim‹, et a pour but d'offrir l'occasion de publier des recherches scientifiques appartenant à la médicine expérimentale et à la biologie étroitement liée avec la médicine.

La revue paraît en cahiers comprenant à peu près 80—100 pages. 3 cahiers forment un volume. Les articles destinés à la revue ne doivent pas être publiés ailleurs. Les manuscrits dactylographiés doivent être rédigés en français, allemand ou anglais et leur longeur totale ne doit pas en général dépasser 24 pages. Un court résumé doit se trouver à la fin.

Le prix de l'abonnement pour la Finlande est marcs 750, pour l'étranger $ 4 ou couronnes suédoises 15 par volume. Les travaux plus étendus, qui seront éventuellement publiés en suppléments, seront distribués gratuitement aux abonnés.

Adresse pour abonnement, échange de journaux et toutes autres communications:

Annales Medicinae Experimentalis et Biologiae Fenniae

Yrjönkatu 17, Helsinki, Finlande.

Annales Medicinae Experimentalis et Biologiae Fenniae

sind eine direkte Fortsetzung der *Acta Societatis Medicorum Fennicae ›Duodecim‹*, 1919—1930 (Vol. I—XII) und der *Acta Societatis Medicorum Fennicae ›Duodecim‹, Ser. A*, 1931—1946 (Vol. XIII—XXIV).

Die Zeitschrift wird vom *Finnischem Ärzteverein ›Duodecim‹* herausgegeben und hat zur Aufgabe wissenschaftliche Untersuchungen aus dem Gebiete der experimentellen Medizin und sich daran schliessenden biologischen Forschungsgebieten aufzunehmen.

Jede Nummer der Zeitschrift erscheint in einem Umfange von 80—100 Druckseiten. Drei Nummern bilden ein Volumen. In die Zeitschrift werden nur Originalarbeiten aufgenommen, die nicht früher veröffentlicht worden sind. Die mit Schreibmaschiene geschriebenen Manuskripte sind in deutscher, englischer oder französischer Sprache einzusenden und sollen im allgemeinen nicht mehr als 24 Druckseiten betragen. Jede Untersuchung ist durch eine kurze Zusammenfassung abzuschliessen.

Der Bezugspreis beträgt für das Innland 750 Fmk und fürs Ausland 4 $ bzw. 15 Schwedenkr. Umfangreichere Untersuchungen, die als Supplemente herausgegeben werden können, erhalten die Abonnenten abgabefrei.

Bezugsadresse sowie Anschrift für Austausch von Zeitschriften und alle anderen Mitteilungen:

Annales Medicinae Experimentalis et Biologiae Fenniae

Yrjönkatu 17, Helsinki, Finnland.

FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF TURKU

DETERMINATION OF SULFONAMIDE SENSITIVITY WITH VARIOUS CULTURE MEDIA

by

SIMO VIRTANEN

(Received for publication May 22, 1951)

Determination of the sulfonamide (SA) sensitivity of bacteria is of great importance in prescribing an appropriate treatment. It is, however, difficult to obtain reliable results owing to the possibility of many technical errors, of which those due to the culture medium are the most difficult to eliminate. Since Lockwood (1938), using peptone, and Woods (1940), with para-amino-benzoic acid (PABA), showed that these substances prevent the bacteriostatic action of SA, many partly or wholly synthetic PABA-free culture media have been used, such as the MacLeod (10) »Liver-infusion medium», Ivanovics (5), Frisk (3), etc. It has been observed, however, that there are many other inhibitors besides PABA. Reports on these are somewhat inconsistent, it is true, obviously because of failure to make the experiments identical. In the following references are made to some of the numerous reports in this domain.

According to MacLeod (10), casein hydrolysates, generally present in synthetic culture media, produced with the aid of acid or alkali hydrolysis do not prevent SA action. However, those produced enzymatically do prevent it, and so do all peptones. Muscle extracts: fresh muscle contained an appreciable amount of inhibitors, but after autolysis the inhibitors increased considerably. Human serum had no inhibiting capacity, whereas some pleural and peritoneal fluids investigated had. Coli bacilli and streptococci contain inhibitors, which are not spontaneously transferred to the

culture medium, but can be freed artificially. Staphylococci and pneumococci, however, release inhibitors into their culture media. Novocaine is a powerful SA antagonist. Finally MacLeod established that both peptone and tissue infusions vary from sample to sample as regards their inhibitor content. Strauss and Finland (17) obtained highly variable results with sulfathiazole and sulfadiazine in different culture media and found that the effect of sulfathiazole was much inhibited in human serum. They agreed in their assertion that the *in vitro* activity does not parallel the *in vivo* experiments. According to West-Coburn (19), coenzymes inhibit the effect of SA, but there are also other antagonists. Ivanovics (5) did not find any inhibitory effect in coenzymes. According to Muir-Shamlefer-Jones (12) there was some inhibiting effect in the casein hydrolysates, of which at least one was an acid hydrolysate. Beef extract and beef serum had no inhibiting effect. According to Lockwood and Fleming, serum does not inhibit the SA effect. Bliss and Long (1941) asserted that methionine and perhaps arginine and lysine have an inhibiting effect. According to Kohn-Harris (7) the sulfonamides prevent synthesis of methionine. Sevag and Green (15) experimented with staphylococci and found an SA-resistent strain to be inhibited if there was no glucose in the culture medium, although it was quite resistent in one containing glucose. They stated that there is no conclusive proof that bacteria synthesize PABA under normal conditions. From the point of view of the action of SA, inhibition of the glucose and the amino acid metabolism would be a critical factor. A synthetic basic medium plus 0.5 per cent glucose plus biotin plus folic acid plus Ca-pantothenate plus pyridoxine did not inhibit the action of SA to any appreciable degree, but if tryptophane was added (1 by 10^{-4} M), the action was almost nonexistent. Without vitamins the antagonistic effect of tryptophane appeared only in the presence of glucose. Sevag and Green also proved that during repeated subcultures (in SA-free media) the sensitivity of the bacteria to SA may change. Clapper-Kurita (1) showed that with *E. coli* and *S. paratyphi* B asparagine, l-cystine and l-tryptophane inhibited the action of SA. Thiamine stimulated it. Riboflavin, nicotinic acid and pyridoxine had no influence, but a mixture of these stimulated *S. paratyphi* B. There is an antagonism between sulfathiazole and methylene blue (2). Riboflavin is an SA antagonist (2).

Besides synthetic culture media, ordinary simple bacteriological culture media were used. As solids these have been used by several workers. Pike-Zimmerman Foster (14) used broth and agar plus 5 per cent rabbit blood without peptone and Jensen-Kiaer (6) agar containing peptone plus 5 per cent horse blood. Sievers (16) also used agar containing 5 per cent horse blood but without peptone. Harper and Cawston (4) found that hemolysed horse blood neutralised SA inhibitors. They used nutrient agar plus 5 per cent horse blood which had either already been hemolysed or was hemolysed in the culture medium by means of incubation and autoclaving. Walker-Philip-Smyth-MacLeod (18) arrived at the same result. Möller (13) did not establish any distinct Harper-Cawston effect, which, according to Harper and Cawston, appears only when horse blood is used and not with human, rabbit or sheep blood. Möller has improved Jensen-Kiaer's method by using peptone-free agar plus 2.5 per cent horse blood plus 0.1 per cent glucose.

In the present investigation comparison between four different solid culture media has been made, by using the so called cup method and the dilution series method.

OWN INVESTIGATION

Material. — The coli bacilli, except for the original D 433, were derived from urine and stool samples. Of the staphylococci used, the strains 209, ^{H}/Oxford and Orion were laboratory control strains, the rest were isolated from samples of pus. After pure culture the strains were kept on the usual slant agar containing peptone. On this medium the control strain Orion was repeatedly cultured during the experiment and other tested strains 0—3 times. The streptococci were isolated from the pharynx swabs of scarlatina patients and kept on the usual agar containing peptone and 5 per cent sheep blood.

Culture Media. — 1. B agar (broth agar) = the usual bacteriological meat extract agar, the agar content being 2 per cent. Before boiling the fresh chopped beef was left standing overnight in + 4° C water. This medium does not contain peptone or glucose, *p*H 7.4.

2. BPe agar (broth peptone agar) = B agar plus 0.5 per cent peptone (peptone Sicc. Orion), *p*H 7.4.

3. BB agar (broth blood agar) = B agar plus 5 per cent fresh defibrinated sheep blood, *p*H 7.4.

4. CPB agar (casein potato blood agar) which has previously been used in Finland by Kokko (8), for instance, and which should be PABA-free. This culture medium is otherwise quite similar to that used by Kokko except that, instead of horse blood, sheep blood was used. The composition of the culture medium was as follows:

I Casein hydrolysate (Orion). — 50 g of casein was hydrolysed according to the method of Ivanovics (5). To a neutral solution was added: 3 g of asparagine, 0.14 g of cysteine, 15 g of sodium citrate, 12 g of glucose, 24 g of NaCl, 0.01 g of ferriammonium citrate, 6 cc of m/1000 aneurin solution and 60 cc m/1000 of nicotinamide solution. The *p*H of the solution was adjusted with NaOH to 7.4. After adding a m/15 phosphate buffer solution (*p*H 7.38) ad 6 litres the solution was dispensed into flasks and sterilised at 105 C for 30 minutes.

II Saline Solution.

$CuSO_4 .5H_2O$ (1 per cent aqueous solution)		5.0 cc
$ZnSO_4 .7H_2O$	»	4.0 »
$MnCl_2.4H_2O$	»	1.5 »
β-alanin		0.115 g
HCl conc.		3.0 cc
H_2O	ad	100.0 »

Sterilised by filtration.

III Potato Broth. — 500 g of cleaned chopped potatoes, 40 cc of glycerin and 1000 cc of distilled water was boiled down into broth and filtered through gauze. The potato broth was prepared separately for each batch of culture medium.

FINAL CULTURE MEDIUM

Casein hydrolysate (solution I)		100.0 cc
Phosphate buffer solution (m/15, pH 7.38)		300.0 »
Tryptophane		0.075 g
Cystine (20 per cent solution)		2.0 cc
Saline solution (solution II)		2.0 »
Potato broth (solution III)	ad	840.0 »
Agar-agar (washed in running water for 24 hours)		20.0 g

A test was made to check that the *p*H had remained unchanged at 7.4; if not, it was corrected. Sterilised for 20 minutes at 120° C, cooled to +56° C, 100 cc of horse serum, 50 cc of defibrinated sheep blood and 10 cc of choline solution (25 mg of choline hydrochloride in 100 cc of distilled water) was added; this final culture medium was kept (mixed occasionally) for 3 hours at +56° C and poured into dishes.

Methods. — Determination of the sensitivity was performed simultaneously on all four culture media. In the dilution series method the testing was done in seven instalments. The meat extract used for a basic medium was prepared separately each time. In the CPB agar the same casein hydrolysate produced by the Orion Pharmaceutical Manufacturers was always used. The potato broth was prepared separately each time. The horse serum and sheep blood were always fresh and the blood was the same as that used in the BB agar. In the cup method the testing was done in five instalments and the culture media likewise prepared separately each time. The Orion staphylococcus was the control in all tests.

The SA preparation used was always Cibazol ampuls containing 20 per cent sulfathiazole. In the Petri dishes the following dilution series were used: 20, 10, 5, 2.5, 1.25, 0.6, 0.3, 0.15, 0.08 mg per cent. sulfathiazole plus the control dish. On the dish there were 5 strains to be tested plus the control strain. In the cup method 30 ml of culture medium was used per dish, the thickness of the agar being 5 mm. For cups porcelain cylinders 5 mm high and with an inner diameter of 1 mm were used. The Cibazol was diluted with saline 1/10, 1/20 and 1/40. Only the inhibition zones of the dilution 1/10 were marked in the table.

For the final inoculation the strains were allowed to grow 24 hours in serum broth, after which the staphylococci were diluted with saline 1/5000, the streptococci 1/1000 and the coli bacilli 1/50000. In the dilution series method the dilution was directly spread with a loop onto the dish. In the cup method 10 cc of the dilution was poured onto the dish and removed with a pipet, after which the dishes were allowed to dry for half an hour open in an incubator. Then the cups were placed on the dish.

The results were read for the staphylococci and the coli bacilli after an incubation of 24 hours (+37° C), for the streptococci after an incubation of 48 hours, since a growth of 24 hours was too slight to be measured.

Results

The results of 12 cultures of staphylococci by means of the dilution series method appear from table 1. The growth was good on all culture media. The cessation of growth was not always abrupt;

TABLE 1 A

INHIBITING CONCENTRATION IN MG PER 100 CC

Strain of Bacteria	B Agar		BPe Agar		BB Agar		CPB Agar	
	P	T	P	T	P	T	P	T
Staphylococc.								
209	—	2.5	—	5.0	—	2.5	—	2.5
H/Oxford	—	2.5	—	5.0	2.5	5.0	5.0	—
Orion	—	2.5	—	5.0	2.5	5.0	5.0	—
1	—	1.25	—	1.25	2.5	5.0	2.5	20
2	—	0.6	1.25	2.5	2.5	5.0	2.5	—
3	—	1.25	10	—	—	2.5	5.0	—
4	—	1.25	10	—	—	2.5	5.0	—
5	—	1.25	10	—	—	1.25	5.0	—
6	—	2.5	—	2.5	—	2.5	5.0	—
7	—	0.6	10	—	0.6	1.25	2.5	—
8	2.5	5.0	—	5.0	5.0	—	5.0	—
9	—	0.08	—	0.3	—	0.15	0.3	—
E. coli								
D/433	1.25	2.5	1.25	2.5	2.5	5.0	1.25	2.5
1	1.25	2.5	—	1.25	2.5	5.0	—	2.5
2	—	1.25	—	1.25	2.5	5.0	—	2.5
3	1.25	2.5	1.25	2.5	—	2.5	1.25	2.5
4	—	0.08	—	0.08	—	0.08	—	0.08
5	1.25	5.0	1.25	2.5	2.5	5.0	2.5	20
6	—	—	—	—	—	—	—	—
7	1.25	2.5	—	1.25	—	1.25	1.25	5.0
8	10	—	10	20	10	—	5.0	10
9 p-coli	1.25	2.5	2.5	5.0	2.5	5.0	—	5.0

P (Partial) = there is still partial growth at the concentration in question
T (Total) = no growth at the concentration in question
— in the column total inhibition concentration
= total inhibition was not obtained at all.
— in the column partial inhibition concentration
= growth ceased abruptly without a partial inhibition area.

prior to cessation a weaker growth could often be observed on some dishes, marked as a partial growth in the table. In the area in question the colonies were considerably smaller, but still visible by the naked eye. In order to obtain a reliable result it would be necessary for growth to cease abruptly, viz. the total inhibition minus the partial inhibition would be 0.

TABLE 1 B

THE RELATION INHIBITION CONCENTRATION: B AGAR TOTAL INHIBITION CONCENTRATION

TABLE 1 C

TOTAL INHIBITION CONCENTRATION MINUS PARTIAL INHIBITION CONCENTRATION (MG PER 100 CC)

B Agar		BPe Agar		BB Agar		CPB Agar		B Agar	BPe Agar	BB Agar	CPB Agar
P	T	P	T	P	T	P	T				
	1		2		1		1	0	0	0	0
	1		2	1	2	2	∞	0	0	2.5	>15
	1			1	2	2	∞	0	0	2.5	>15
	1		1	2	4	2	16	0	0	2.5	17.5
	1	2	4	4	8	4	∞	0	1.25	2.5	>17.5
		8	∞		2	4	∞	0	>10	0	>15
	1	8	∞		2	4	∞	0	>10	0	>15
	1	8	∞		1	4	∞	0	>10	0	>15
	1		1		1	2	∞	0	0	0	>15
	1	16		1	2	4	∞	0	>10	0.6	>17.5
½	1	1	1	1	∞	1	∞	2.5	0	>15	>15
	1		4		2	4	∞	0	0	0	>20
½	1	½	1	1	2	½	1	1.25	1.25	2.5	1.25
½	1		½	1	2		1	1.25	0	2.5	0
	1		1	2	4		2	0	0	2.5	0
½	1	½	1		1	½	1	1.25	1.25	0	1.25
	1		1		1		1	0	0	0	0
¼	1	¼	½	½	1	½	4	3.75	2.5	2.5	17.5
½	1		½		½	½	2	1.25	0	0	3.75
∞	∞	∞	∞	∞	∞	∞	∞	>10	10	>10	5.0
½	1	1	2	1	2		2	1.25	2.5	2.5	0

B agar = broth agar
BPe » = » peptone agar
BB » = » blood agar (5%)
CPB » = casein potato blood agar
> = over
∞ = uncertain quantity (number)

This is the case with B agar in 11, with BPe agar in 7, with BB agar in 6 and with CPB agar in 1 out of 12 cases. A total inhibition was recorded with B agar 12 times, with BPe agar 7, with BB agar 11 times and with CPB agar once only (table 1 A and C). The inhibiting concentrations on other culture media tended to be higher than on B agar (table 1 B).

TABLE 2 A

DIAMETER OF INHIBITION ZONE IN MM

Strain of Bacteria	B Agar		BPe Agar		BB Agar		CP B Agar	
	P	T	P	T	P	T	P	T
Staphylococc.								
209	38	36	30	27	26	25	23	21
H/Oxford	35	34	32	30	21	20	22	—
Orion	37	36	27	26	26	26	26	—
1	36	36	32	32	27	27	28	28
2	37	37	31	28	23	20	25	—
3	30	30	28	28	26	25	26	—
4	32	32	27	27	21	21	23	—
5	31	31	28	27	25	25	23	—
6	25	23	22	20	20	19	15	—
7	37	31	35	30	24	23	26	—
8	23	22	22	20	20	18	18	—
9	60	60	50	50	47	47	35	35
E.coli								
D/433	33	30	30	25	30	23	28	25
1	33	26	30	23	31	24	29	—
2	32	25	32	25	32	24	29	—
3	31	28	29	20	29	26	25	25
4	40	40	40	35	39	29	45	33
5	30	—	27	—	28	—	27	—
6	40	—	32	—	30	—	33	—
7	35	25	31	23	32	23	31	26
8	26	—	27	—	25	—	24	—
9 p-coli	31	31	17	15	17	17	18	18

P = Partial inhibition zone
T = Total » »
— in the column total inhibition zone = total inhibition was not obtained at all.

The coli strains (10) all grew well. No. 6 is obviously a very resistant strain since it »grew through». The differences between the various culture media are rather small. BPe agar is even somewhat better than B agar. BB agar and CPB agar are a little less good.

In the cup method the diameter of the inhibition zones was measured in two directions at right angles and the mean value was

TABLE 2 B

DIFFERENCE AS COMPARED WITH B AGAR IN MM

TABLE 2 C

DIAMETER OF PARTIAL INHIBITION ZONE MINUS DIAMETER OF TOTAL INHIBITION ZONE IN MM

B Agar		BPe Agar		BB Agar		CPB Agar		B Agar	BPe Agar	BB Agar	CPB Agar
P	T	P	T	P	T	P	T				
0	0	— 8	— 9	—12	—11	—15	—15	2	3	1	2
0	0	— 3	— 4	—14	—14	—13	—34	1	2	1	22
0	0	—10	—10	—11	—10	—11	—37	1	1	0	26
0	0	— 4	— 4	— 9	— 9	— 8	— 8	0	0	0	0
0	0	— 6	— 9	—14	—17	—12	—37	0	3	3	25
0	0	— 2	— 2	— 4	— 5	— 4	—30	0	0	1	26
0	0	— 5	— 5	—11	—11	— 9	—32	0	0	0	23
0	0	— 3	— 4	— 6	— 6	— 8	—31	0	1	0	23
0	0	— 3	— 3	— 5	— 4	—10	—23	2	2	1	15
0	0	— 2	— 1	—13	— 8	—11	—31	6	5	1	26
0	0	— 1	— 2	— 3	— 4	— 5	—22	1	2	2	18
0	0	—10	—10	—13	—13	—25	—25	0	0	0	0
Average		—4.8	—5.3	—9.6	—9.3	-10.9	—27	1.1	1.8	0.8	17.2
0	0	— 3	— 5	— 3	— 7	— 5	— 5	3	5	7	3
0	0	— 3	— 3	— 2	— 2	— 4	—26	7	7	7	29
0	0	0	0	0	— 1	— 3	—25	7	7	6	29
0	0	— 2	— 8	— 2	— 2	— 6	— 3	3	9	3	0
0	0	0	— 5	— 1	—11	+ 5	— 7	0	5	10	15
0	0	— 3	0	— 2	0	— 3	0	30	27	28	27
0	0	— 8	0	—10	0	— 7	0	40	32	30	33
0	0	— 4	— 2	— 3	— 2	— 4	+1	10	8	9	5
0	0	+ 1	0	— 1	0	— 2	0	26	27	25	24
0	0	—14	—16	—14	—14	—13	—13	0	2	0	0
Average		—3.6	—3.9	—3.8	—3.9	—4.2	—8.0	12.6	12.9	12.5	16.5

entered in the table. Outside the inhibition zone a pale area was generally observed on which growth was denser than usual [as, for instance, Lamanna-Shapiro (9)]. Inside it growth ceased more or less abruptly.

For the staphylococci the average difference between the partial and total inhibition on B agar was 1.1 mm, on BPe agar 1.6 mm, on BB agar 0.8 mm, and on CPB agar 17.2 mm (table 2 C), on which

TABLE 3 A

INHIBITING CONCENTRATION IN MG PER 100 CC

Strain of Bacteria	B Agar		BPe Agar		BB Agar		CPB Agar	
	P	T	P	T	P	T	P	T
Streptococc.								
1 α-haemolytic. .	*0.08	0.6	*0.15	20	—	—	—	—
2 » ..	*0.08	0.6	*0.15	0.6	—	1.25	1.25	2.5
3 » ..	*0.08	0.15	*—	0.6	—	1.25	1.25	5.0
4 β- » ..	*—	0.6	*—	2.5	—	5.0	—	20
5 » ..	*0.08	0.15	2.5	10	1.25	5.0	*5.0	20
6 » ..	**		*—	0.3	5.0	10	**	
7 » ..	*0.15	0.6	*0.3	5.0	20	—	*0.08	1.25
8 » ..	0.6	1.25	5.0	10	—	—	—	—
9 » ..	*0.08	0.6	*—	—	2.5	—	*0.15	2.5
10 » ..	0.3	5.0	—	—	2.5	—	5.0	—
11 » ..	*0.3	2.5	*—	—	2.5	—	5.0	—
Control strain Orion in the different experiments	—	1.25	—	2.5	—	1.25	2.5	—
	—	1.25	10	—	—	2.5	5.0	—
	—	2.5	5.0	—	—	2.5	5.0	—
	1.25	2.5	5.0	20	2.5	5.0	20	—
	—	2.5	—	5.0	2.5	5.0	5.0	—
	—	1.25	1.25	2.5	2.5	5.0	2.5	5.0
	—	1.25	1.25	2.5	2.5	5.0	2.5	20

* = poor growth in controls, too
** = no growth

a total inhibition was obtained in only 3 cases (table 2 A). The inhibition zones of the other culture media were considerably smaller than those obtained on B agar (table 2 B).

With coli bacilli the partial minus total inhibition were of almost the same degree on B, BPe and BB agar (table 2 C). Here also the inhibition zones of the other culture media were smaller than on B agar (table 2 B).

Of 11 strains of streptococci, 9 on B agar, 7 on BPe agar and 4 on CPB agar grew sparsely or not all (table 3 A). Therefore no comparison was made as in the case of staphylococci and coli bacilli. The

TABLE 3 B

DIAMETER OF INHIBITION ZONE IN MM

B Agar		BPe Agar		BB Agar		CPB Agar	
P	T	P	T	P	T	P	T
*		*		—	—	—	—
*		*		—	30	28	—
**		**		—	20	22	—
*		*		35	—	32	—
*		27	—	20	—	*	
*21	—	*		—	—	—	—
*		15	—	—	—	—	—
**		*		29	—	*	
*		28	—	31	—	—	—
*		*		24	—	—	—
30	26	29	25	26	24	20	—
31	28	27	25	28	28	22	—
28	28	27	25	26	26	24	—
38	38	35	32	27	27	25	—
28	28	25	—	23	23	21	—

growth on BB agar was good in all cases. With 5 strains a total inhibition was obtained, with 4 a partial and with 2 no inhibition. In the cup method the thin growth was a further difficulty, except with BB agar (table 3 B). On BB agar a distinct inhibition zone was only obtained with 2 strains, with 5 strains a partial inhibition was obtained and in the inhibition area of the latter hemolysis did not occur and the colonies were smaller than elsewhere. In 3 cases there was no inhibition at all.

The terms partial and total inhibition refer to the situation at the moment of inspection only. Evidently even on an area of total

inhibition the bacteria survive and are capable of multiplying when transferred to an SA-free medium. If the period of incubation is prolonged, a growth may gradually be seen on a previously empty area.

The results obtained with the control strains (table 3 A and B) show considerable differences even between the various batches of the same culture medium. This is most obvious with BPe agar containing peptone, with which a few times there was no total inhibition at all. With CPB agar total inhibition was almost entirely absent. Therefore even the results determined according to the same method and with the same culture medium are not strictly comparable with one another.

SUMMARY

1. Simple peptone-free meat extract agar gave the best results with staphylococci and coli bacilli. The different batches of culture medium gave somewhat varying values.

2. As such, this culture medium is too poor for streptococci. By adding 5 per cent sheep blood a medium is obtained which is satisfactory for streptococci too. The blood neutralizes to some extent the SA effect, but the results are still utilizable.

3. Agar containing peptone contains SA inhibitors which vary considerably in quantity — so much, in fact, that the results cannot be considered reliable.

4. The partly synthetic CPB agar proved less useful because the inhibition zone was hard to determine.

REFERENCES

1. Clapper, W. E., and Kurita, I. T.: J. of Bact. 1948:*55:*277.
2. Gots, J. S., and Sevag, M. G.: J. of Bact. 1949:*58:*585.
3. Frisk, R.: Acta Med. Scand. Suppl. 1943:142.
4. Harper, G. J., and Cawston, J.: J. of Path. & Bact. 1945:*57:*59.
5. Ivanovics, G.: Ztschr. f. Immunitätf. 1942:*102:*58.
6. Jensen, K. A., and Kiaer, L.: Acta Path. & Microbiol. Scand. 1948: *25:*146.
7. Kohn, H. I., and Harris, J. S.: J. of Bact. 1942:*44:*717.
8. Kokko, U. P.: Annal. Med. Exp. & Biol. Fenn. 1947:*25:*79.
9. Lamanna, C., and Shapiro, I. M.: J. of Bact. 1943:*45:*385.
10. MacLeod, C. M.: J. of Exp. Med. 1940:*72:*217.

11. MacLeod, C. M., and Mirick, G. S.: J. of Bact. 1942:*44:*277.
12. Muir, R. D., Shamleffer, V. J., and Jones, L. R.: J. of Bact. 1942: *44:*95.
13. Möller, O.: Svenska Läkartidn. 1949:*31:*1623.
14. Pike, R. M., and Zimmerman Foster, A.: J. of Bact. 1944:*47:*97.
15. Sevag, M. G., and Green, M. N.: J. of Bact. 1944:*47:*450 and J. of Bact. 1944:*48:*615.
16. Sievers, O.: Svenska Läkartidn. 1948:*35:*1650.
17. Strauss, E., and Finland, M.: Proc. Soc. Exp. Biol. & Med. 1941:*67:* 428.
18. Walker, N., Philip, R., Smyth, M. M., and MacLeod, J. W.: J. of Path. & Bact. 1947:*59* :631.
19. West, R., and Coburn, A. F.: J. of Exp. Med. 1940:*72:*91.

FROM THE DEPARTMENT OF PHYSIOLOGY, AND THE CHILDREN'S CLINIC,
UNIVERSITY OF HELSINKI

THE SOLUBILITY OF HAEMOGLOBIN AND THE INTRACELLULAR ELECTROLYTES OF THE ERYTHROCYTES OF DIFFERENT SHEEP

by

M. J. KARVONEN and V. LEPPÄNEN

(Received for publication May 30, 1951)

In a study of the solubility of adult and foetal sheep haemoglobin (Hb), it was observed that the Hb of adult sheep available at Cambridge had quite different solubility characteristics than adult sheep Hb obtainable in Helsinki (6). The *p*H-solubility curve of the Hb of the Cambridge sheep resembled that of horse Hb, as described by previous investigators (3, 9, 10), having two minima in the region of *p*H 5.5 and 6.5, respectively. The Helsinki variety of the adult Hb, however, showed the smaller solubility the more alkaline the *p*H, within the range of the ammonium phosphate buffer used, i.e. between *p*H 4.5 and 7.1, and it had no minimum in this range. Above *p*H 6 or 6.5, the precipitate of the adult haemoglobins also showed different behaviour, the Cambridge variety precipitating as rhomboid plates and the Helsinki type as needles. At lower *p*H:s, the crystals of both assumed the plate-shape. (The crystallography of the plate-shaped crystals of sheep Hb: see (7). It appeared that the two types of crystals were not thermodynamically equivalent, as the needles were not stable at an acid *p*H. Both types of crystals are to be seen in Fig. 1.

In Helsinki, samples of post-natal Hb obtained from two sheep were examined, both showing the above behaviour. However, at Cambridge one lamb was found, whose Hb at repeated samplings exhibited similar solubility characteristics as observed later on in the Helsinki series, but this was the only exception in a series of samples taken from 26 adult sheep and lambs.

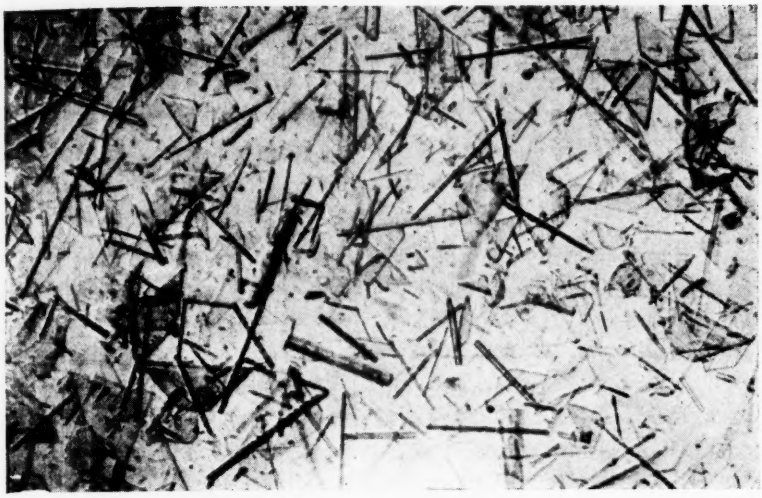


Fig. 1. — Two types of crystals of sheep adult haemoglobin. The mixture of crystals results from a precipitation at a »critical» *p*H, 6.5 (20×.)

In view of this evidence, it was difficult to regard these two adult sheep Hb:s as identical substances. However, the situation was complicated by the finding that in a constant solvent solubility test, each of these Hb:s behaved as a two-component system. According to a graphic analysis of the constant solvent solubility diagrams, none of the supposed four components had an identical solubility, but in each case the components of the original mixture — if this was not an artifact — seemed to have rather similar *p*H-solubility relations as the resulting mixture. Therefore, it seemed that the observed differences do not simply depend on the presence of a mixture of two compounds at a varying proportion. However, errors may be produced in the interpretation of solubility diagrams by various interactions between the proteins of the system (6). Solubility experiments with artificial mixtures may be expected to be more helpful in an attempt to prove the non-identity of two proteins. In the aforementioned study, this was not done, because enough of suitable material was not simultaneously available.

At the time of the above study, no suggestion could be made as to the cause of the different behaviour of the two adult sheep Hb:s. Meanwhile, however, Hallman and Karvonen (4) observed that the intracellular electrolytes of the red cells of the Finnish breed of

sheep were different from corresponding values previously published in other parts of the world. Thus, it seems that adult sheep of different breeds may show intracellular sodium values varying between 16 (8) and 89 (1) millieq. per kg, and potassium values between 103[1] (4) and 16 (1) millieq., respectively.

The present investigation is an attempt to study the intracellular electrolytes and the *p*H-solubility curves of the Hb of adult sheep blood obtained from sheep belonging to various breeds. It was thought that such a study might offer a clue to the cause of the difference observed between the two adult Hb:s. In fact, it turned out that a clearcut correlation obtained between the intracellular electrolytes and the solubility of Hb found in the group of samples studied, but that the difference in solubility was not probably due to a basic difference in the haemoglobins.

MATERIAL AND METHODS

Material. — Five samples of adult sheep erythrocytes were examined. The sources of the samples were as follows:

No.	*Sex*	*Breed*	*Sheep Kept at*
1	Ewe	Clun Forest Breed	Institute of Animal Physiology, Babraham, Cambs., England.
2	Ram	Cheviot	The Rowett Research Institute, Aberdeenshire, Scotland
3	Ewe	Blackface	»
4	Ram	Dorset Horn	»
5	Ewe	Finnish Country Sheep	University Farm, Helsinki, Finland.

Blood was drawn under sterile conditions from the jugular vein. The coagulation was prevented in samples No. 1 and 5 by means of heparin, and in samples 2, 3, and 4 by using potassium oxalate. The samples were immediately centrifuged, and the plasma was discarded. A portion of the corpuscle mass was bottled as such for the electrolyte determinations, and the rest was washed with saline at least twice, treated with coal gas, and bottled. The samples from Great Britain were sent by air mail to Helsinki, where the subsequent operations were carried out.

Electrolyte Determinations. — The concentrations of Na and K

[1] This figure is actually millieq. per litre.

in the corpuscle mass were determined with a flame photometer (5), after the preliminary precipitation of proteins with nitric acid. The potassium values of the samples No. 2, 3, and 4 are too high because potassium oxalate was used as an anticoagulant; a measure of the relative magnitude of the error was obtained by determining the Ca-oxalate and total oxalate concentration of these samples. [Method: (2)].

Solubility Studies. — The Hb solutions were prepared as 5 per cent carboxy-haemoglobin, by laking the washed corpuscles with distilled water. Stroma was removed by filtering with siliceous earth, and by precipitating the dissolved non-Hb proteins by adding a weighed amount of ammonium sulphate to bring its concentration to $\Gamma/2 = 4.5$. The solution was again cleared by adding a small amount of siliceous earth and by filtering with suction through a Whatman No. 42 paper. The clear solutions were diluted with an equal volume of $\Gamma/2 = 4.5$ $(NH_4)_2SO_4$, and precipitated slowly by dialysis in cellophan tubes against gradually rising concentrations of $(NH_4)_2SO_4$. Thus performed, the precipitation was found to occur at approcimately *p*H 6. In some experiments, the precipitation was carried out in a buffered medium, at the *p*H desired. After the samples had been brought into equilibrium with $\Gamma/2 = 9.0$ $(NH_4)_2SO_4$, they were centrifuged, washed three times with ammonium sulphate of the same strength, and finally resuspended in this medium to make the concentration of the CO-Hb to 2 per cent.

For studying the *pH solubility curves* of the samples, 2.0 ml aliquots of unbuffered, homogenized CO-Hb suspension were pipetted into a series of small (capacity appr. 5 ml) test tubes containing 2.0 ml of a mixture of two solutions, A and B. These were: A, a $\Gamma/2 = 4.0$ $(NH_4)_2SO_4$ solution made into a 0.4 molar $(NH_4)_2HPO_4$ buffer, and B, a solution of the same strength, but made into $NH_4H_2PO_4$. The proportion of A and B was varied according to the *p*H desired.

In order to study the *identity versus non-identity* of the samples of Hb, series of artificial mixtures were made of them. Five tubes with identical buffer and salt concentrations were used for each comparison. Two of them represent samples as treated above. To another two of the five tubes, 2.0 ml of a 1 per cent CO-Hb suspension in $\Gamma/2 = 9.0$ $(NH_4)_2SO_4$ were added, instead of the 2 per

cent suspension generally used. To the fifth tube, 1.0 ml of each CO-Hb was added as a 2 per cent suspension.

The tubes were filled with coal gas and closed with rubber stoppers. The equilibration between the protein in solid phase and solution was brought about by stirring the tubes for 24 hrs in a Wifug shaking machine, and by allowing them to stand after that at least for 48 hrs. After that, the tubes were centrifuged in an angle sentrifuge, the supernatant CO-Hb solution was pipetted off, cleared with the aid of another centrifugation, if necessary, and the concentration of CO-Hb was determined in a Lumetron electrophotometer, at 550 $\mu\mu$, generally using an optic depth of 10 mm.

All the operations except the photometric determinations were carried out at $+ 0.5 \pm 0.3°$ C, in a refrigerator room, and the samples were continuously kept under a coal gas atmosphere.

A portion of the precipitate was examined by using an ordinary microscope.

RESULTS

Intracellular Electrolytes. — The results of the electrolyte analyses are presented in Table I. It must be noted that the potassium values of the samples No. 2, 3, and 4 are too high because of the addition of potassium oxalate. It was not possible to calculate

TABLE I

THE INTRACELLULAR CONCENTRATION OF SODIUM AND POTASSIUM IN THE ERYTHROCYTES OF FIVE SHEEP OF DIFFERENT BREEDS. THE VALUES FOR POTASSIUM IN SAMPLES 2, 3, AND 4 ARE TOO HIGH BECAUSE POTASSIUM OXALATE HAD BEEN USED AS AN ANTICOAGULANT. THE CALCULATION OF THE CORRECTED VALUES IS EXPLAINED IN THE TEXT

No.	Na Millieq. per Litre	K Millieq. per Litre	
		No Correction	Corrected
1	117	22.0	
2	113	(36.8)	17
3	60.0	(130)	70
4	112	(40.0)	18
5	34.0	87.1	

an exact correction for these figures, because of the many uncontrolled factors, but an approximate correction was made as follows. It was assumed that the sum of the — probably correct — sodium and the corrected potassium values would be 130 millieq. per litre. The difference between this figure and the sodium values, i.e. the

corrected potassium value, is shown in Table I. According to this method of calculation, the correction is greatest for sample No. 3. This was found justified, as this sample was observed to contain twice the amount of dissolved oxalate than samples No. 2 and 4.

These figures, even if they may not be quite correct, nevertheless indicate clearly that samples No. 1, 2, and 4 represent »sodium-type» erythrocytes, whereas samples No. 3 and 5 are characterized by a fairly high intracellular potassium concentration. The differ-

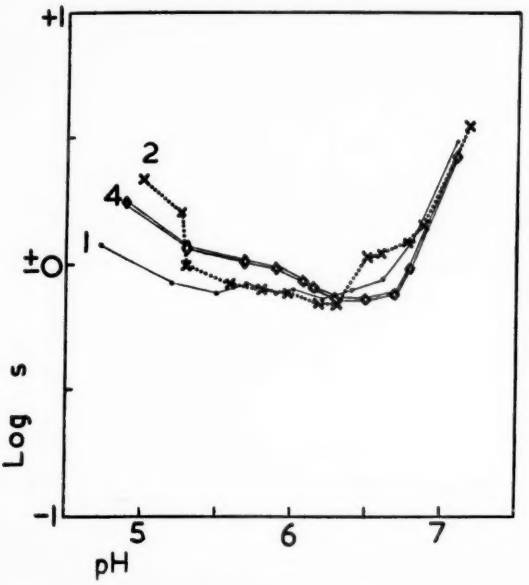


Fig. 2. — The logarithm of the solubility of Hb, as plotted against the *p*H. Samples No. 1, 2, and 4. Ionic strength: 7.50. Equilibrium approached from the undersaturated side; precipitate »plates».

ence between the sodium values of the samples No. 1, 2, and 4 are rather small, and these three samples obviously form a homogeneous group. The intracellular sodium concentration of the sample No. 3 is intermediate to the above group and sample No. 5, but evidently too far from either to be joined with them.

The Solubility of the Haemoglobins. — When the five samples of Hb were precipitated with $(NH_4)_2SO_4$ approximately at *p*H 6, without phosphate buffer, the samples No. 1, 2, and 4 assumed the crystal shape of »plates» known from the Cambridge sheep, sample No. 3 precipitated as »needles», and sample No. 5 as an amorphous

precipitate. Samples No. 1, 2, and 4 showed no change of the precipitate during the equilibration at the various *p*H:s used. Their *p*H-solubility curves are shown in fig. 2. However, the precipitate of samples No. 3 and 5 was completely dissolved at *p*H:s below 6, and a precipitation as »plates» ensued. In these both series, the equilibrium between the protein in the solid phase and in the solution was thus approached from the supersaturated side below

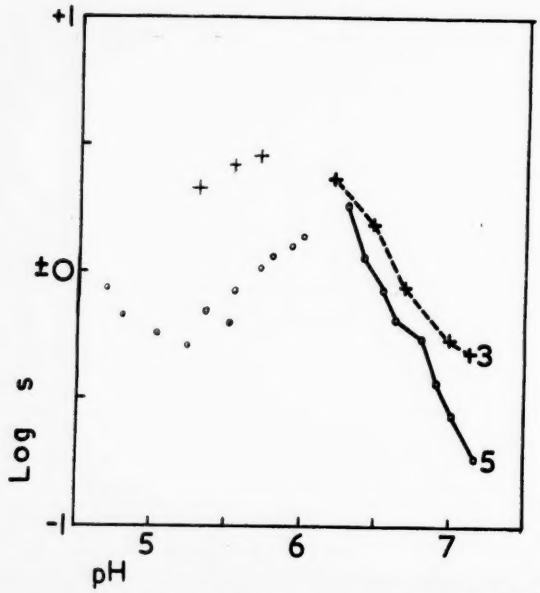


Fig. 3. — Samples No. 3 and 5. Ionic strength: 7.50. Equilibrium approached from the undersaturated side, by using a precipitate of »needles». Below *p*H 6 these proved unstable, and a precipitation as »plates» ensued.

*p*H 6. Sheep haemoglobins — like many proteins — are known to have a tendency to remain in solutions supersaturated as referred to the solubility of the corresponding crystals, even in the presence of a crystalline solid phase (6). Therefore, the solubility values for the samples No. 3 and 5 (Fig. 3) below *p*H 6 are not directly comparable with solubility values attained by approaching the equilibrium from the undersaturated side, as in samples No. 1, 2, and 4 (Fig. 2).

For making it possible to compare the *p*H-solubility curves, samples No. 3 and 5 were precipitated at a *p*H low enough to yield a precipitate of »plates»; the precipitate was washed as usually and its solubility was studied at a few *p*H:s. The results are shown in

Fig. 4. Thus these two samples of haemoglobin showed solubility characteristics very similar to those of the samples No. 1, 2 and 4, which primarily precipitated as »plates».

A number of artificial mixtures of two haemoglobins was made by using the samples as various precipitates. It was expected that if the different samples contain different haemoglobins, the solubility of the mixture would exceed that of the components.

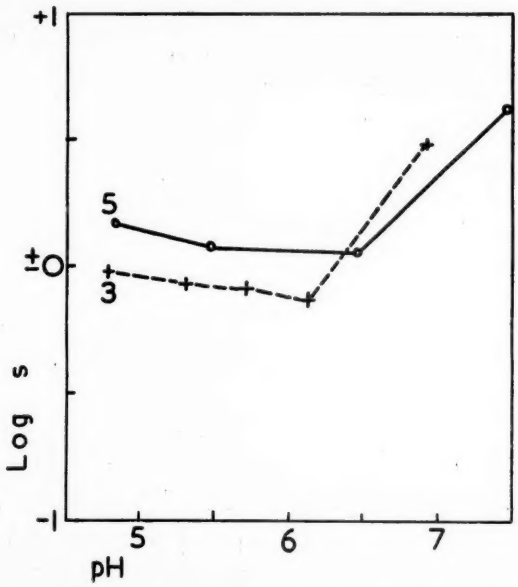


Fig. 4. — Samples No. 3 and 5. Ionic strength: 7.50. Equilibrium approached from the undersaturated side, by using a precipitate of »plates».

It was first ascertained that reproducible solubility values which were compatible with the requirements of a thermodynamical equilibrium were attainable, only if the equilibrium was consistently approached from the undersaturated side. If the starting material represented a solid phase unstable at the *p*H of the mixture, obviously irregular solubility values were often obtained (Table II). For instance, the solubility anomalously showed an increase with a decrease in the total protein concentration (Table II, series 1, 2, 4, 10, 12, 13, 15). Similarly, when the solubility of a mixture exceeded the sum of the solubilities of the components (Table II: series 13), a thermodynamical equilibrium was obviously not attained.

TABLE II

EXAMPLES OF THERMODYNAMICALLY »IRREGULAR» SOLUBILITY VALUES OBTAINED ON ARTIFICIAL MIXTURES OF DIFFERENT SAMPLES OF SHEEP HAEMOGLOBIN. THE STARTING MATERIAL WAS A SOLID PHASE UNSTABLE AT THE *p*H OF THE FINAL EQUILIBRATION. AM = PRECIPITATE AMORPHOUS; N = PRECIPITATE »NEEDLES»; P = PRECIPITATE »PLATES»

No.	Mixture A	Mixture B	*p*H	Solubility g. Hb per 1000 ml & Solid Phase 1 % A	0.5 % A	0.5 % A— 0.5 % B	0.5 % B	1 % B
1	3N	5Am	7.00	0.64N	0.63N	0.65N	0.70N	0.67N
2	»		5.95	3.16N&P	4.08N&P	4.08N&P	2.64N	4.46N
4	4P	5Am	7.00	2.23P	1.89P	0.60N&P	0.60N	0.58N
5	»		5.95	0.60P	0.59P	1.50N&P	2.64N	4.46N
10	3P	5Am	7.00	1.00N	1.04N	1.12N	1.89N	1.46N
12	»		5.00	0.79P	0.73P	0.76P	0.46P	0.43P
13	1P	5Am	7.00	1.17P	1.11P	4.33P&N	1.89N	1.45N
15	»		5.00	1.36P	1.17P	1.04P	0.46P	0.43P

TABLE III

THERMODYNAMICALLY REGULAR SOLUBILITY VALUES OBTAINED ON ARTIFICIAL MIXTURES OF DIFFERENT SAMPLES OF SHEEP HAEMOGLOBIN. THE STARTING MATERIAL WAS A SOLID PHASE STABLE AT THE *p*H OF THE FINAL EQUILIBRATION. P = PRECIPITATE »PLATES»

No.	Mixture A	Mixture B	*p*H	Solubility g. Hb per 1000 ml & Solid Phase 1 % A	0.5 %A	0.5 % A— 0.5 % B	0.5 % B	1 % B
16	1P	2P	7.00	1.17P	1.11P	1.14P	0.98P	1.09P
17	»		6.10	0.75P	0.67P	0.74P	0.71P	0.78P
18	»		5.00	1.36P	1.17P	1.61P	1.31P	1.47P
19	2P	4P	7.00	1.09P	0.98P	1.19P	1.04P	1.17P
20	»		6.10	0.78P	0.71P	0.72P	0.66P	0.73P
21	»		5.00	1.47P	1.31P	1.53P	1.34P	1.51P

Definite value could be given only to such equilibrations, in which the crystal habit of the precipitate remained unchanged during the equilibration, and where, of course, the samples to be compared were treated as identically as possible. For this purpose, the precipitation was carried out at the same *p*H as the final equilibration. Table III shows the results of such series (Nos. 16—21). The results show that the solubility of the artificial mixture generally comes quite close to that of the original samples at the same total protein concentration.

In the artificial mixtures Nos. 23—25 (Table IV), mixtures of

TABLE IV

SOLUBILITY VALUES OBTAINED ON ARTIFICIAL MIXTURES OF DIFFERENT SAMPLES OF SHEEP HEAMOGLOBIN AT CRITICAL *p*H:S WHERE BOTH TYPES OF CRYSTALS WERE EXPECTED TO BE ALMOST EQUALLY STABLE. N = PRECIPITATE »NEEDLES»; P = PRECIPITATE »PLATES»

No.	Mixture A	Mixture B	*p*H	Solubility g. Hb. per 1000 ml & Solid Phase 1 % A	0.5 % A	0.5 % A— 0.5 % B	0.5 % B	1 % B
22	4P	5P	6.85	1.43P	1.26P	1.30P	1.28P	1.66P
23	3N	4P	6.85	0.36N	0.32N	1.09N&P	1.20P	1.43P
24	»		6.40	0.60N	0.57N	0.85N&P	0.76P	0.94P
25	3N	5P	6.85	0.36N	0.32N	1.06N&P	1.28P	1.66P

»needles» and »plates» were studied at critical *p*H:s where both types of crystals were expected to be almost equally stable (compare Figs. 2, 3, and 4). In these cases, the solubility of the mixture was intermediate to that of the pure components at the same total protein concentration. The interpretation of such a result is dealt with in »Discussion».

DISCUSSION

The solubility relations of each of the samples examined differed from those of a pure protein; when the amount of protein precipitate was increased in the system, the amount of Hb dissolved showed a rise (Table III and IV). This is in agreement with previous results (6) on the solubility of sheep adult Hb.

Consistent differences obtained in the tendency of the different samples to assume either the »plate» or the »needle» crystal habit. Samples 1, 2, and 4 never crystallized in the latter shape within the range of *p*H used, but samples 3 and 5 assumed this shape at sufficiently high *p*H:s. At lower *p*H:s, all samples precipitated in an apparently identical manner, as »plates». The different behaviour was not abolished by dialysis, recrystallization or keeping of the samples. Such a variation in the crystal habit may, of course, depend on a difference in the haemoglobins themselves or on an uncontrolled impurity following the Hb.

The solubility studies on artificial mixtures suggest that one is not dealing with basically different types of Hb. If it were so, it would be expected that the solubility of the artificial mixtures would be of the order of the sum of the solubilities of the components, and this did not occur in the present material. As the com-

ponents of these artificial mictures were not pure single proteins each, the possibility remains that the difference in crystal habit is due to the presence of a mixture at varying proportions of two or more closely related species of Hb molecules.

The solubility values of the artificial mixtures 23—25 as presented in Table IV were intermediate to those of the components of a mixture. Such a finding may be given two explanations. Either the less soluble solid phase has acted as a nucleus for the precipitation of molecules dissolved from the more soluble solid, or the results represent a thermodynamical equilibrium characterized by the presence of a solid solution as the solid phase. The latter possibility occurs when mixtures — naturally occurring or artificial — of sheep foetal and adult Hb are precipitated (6). However, the resulting solid phase has often a quite characteristic appearance, differing from each of the components. In the present work, this was not observed. Thus, it is more likely that the intermediate solubilities simply depended on the instability of the more soluble form of precipitate.

Whatever may be the factor causing the observed differences in the crystal habit, it seems to be in a close relation to the composition of the intracellular electrolyte medium of the erythrocytes. The distribution of the samples according to their crystal habit is the same as according to the intracellular sodium concentration.

The hypothesis is near that the intracellular electrolyte medium conditions the Hb synthesis in the red cell, and that the type of Hb or of Hb mixture produced depends on the intracellular electrolytes, either directly or indirectly. The present work does by no means prove such a hypothesis; more observations and experimental work is needed for that. However, in the present material this is the only covariation, as there is no regularity according to the diet or to the place of origin of the samples. Whether the variation of the intracellular electrolytes and of the characteristics of the Hb depend on genetic variations or on some physiological variables, can not be decided upon the present five cases.

SUMMARY

1. The intracellular Na and K concentrations of 5 samples of sheep erythrocytes obtained from different sources were determined. These values were compared with the solubility and crystal habit of the haemoglobin (Hb) of each sample.

2. The intracellular Na values varied between 34.0 and 117 milleq. per litre in the different samples.

3. Those two samples showing the lowest intracellular Na concentration contained Hb which precipitated at *p*H 7 in crystals (»needles») differing in shape and thermodynamical characteristics from those of the same samples as precipitated at *p*H:s below 6; the latter type of crystals (»plates») was the only crystal habit the three other samples assumed within the range of the buffer employed.

4. The solubility of artificial mixtures of Hb prepared from the different samples was in general of the same order as the solubility of the components of the mixture. The solubility of the original sample increased with the total Hb concentration of the system, thus suggesting the presence of a mixture of proteins.

5. The results were interpreted as suggesting that there is in the low-sodium sheep erythrocytes some factor, either a difference in the composition of the Hb mixture or some uncontrolled impurity, which causes the different crystal habit of the samples.

Acknowledgements. — The writers are greatly indebted to Professor I. DE BURGH DALY, from the Institute of Animal Physiology, and to Dr. A. T. PHILLIPSON, from the Rowett Research Institute, for taking blood samples and sending them duly prepared to us. The writers have also been aided by a personal grant from »Lastentautien Tutkimussäätiö» (Foundation for the Investigation of Children's Diseases).

REFERENCES

1. ABDERHALDEN, E.: Z. physiol. Chem. 1898:*25:*65.
2. CLARK, E. P., and COLLIP, J. B.: J. biol. Chem. 1925:*63:*461.
3. GREEN, A.: J. Biol. Chem. 1931:*93:*517.
4. HALLMAN, N., and KARVONEN, M. J.: Ann. Med. Exper. Biol. Fenn. 1949:*27:*221.
5. HALLMAN, N., and LEPPÄNEN, V.: Suomen Kemistilehti B 1949:*22:*55.
6. KARVONEN, M. J.: Thesis. Cambridge 1949.
7. KENDREW, J. C., and PERUTZ, M. F.: Proc. Roy. Soc. A 1948:*194:*375.
8. KERR, S. E.: J. Biol. Chem. 1937:*117:*227.
9. SØRENSEN, S. P. L., and SØRENSEN, M.: Meddelelser fra Carlsberg Lab. 1930:*19:*No. 11.
10. SØRENSEN, S. P. L., and SØRENSEN, M.: Biochem. Z. 1933:*258:*16.

FROM THE DEPARTMENT OF PHYSIOLOGY AND THE CHILDREN'S CLINIC,
UNIVERSITY OF HELSINKI

A NOTE ON THE SUPERSATURATION OF PROTEIN SOLUTIONS

by

M. J. KARVONEN and V. LEPPÄNEN

(Received for publication May 30, 1951)

In the early studies of the solubility of proteins, an anomalous behaviour was often observed. It was found that, under otherwise equal circumstances, the protein was salted out the more completely, the higher the starting protein concentration. This type of solubility curve was observed e.g. by Chick and Martin (2) in the course of their studies on egg albumin. Sørensen (4), however, explained the occurrence of such solubility curves as being due to the uneven distribution of water and the precipitating salt between the mother liquor and the crystal water. The precipitation resulted in an increase of the electrolyte concentration in the mother liquor, which again led to a decrease of the solubility. In subsequent studies, solubility curves of a corresponding shape have been ascribed to the same cause, without actual electrolyte determinations, e.g. in a study of the solubility of adult and foetal cattle haemoglobin (Hb) by Wyman, Raffert and Ingalls (5), where a further increase of the protein concentration in the system beyond the beginning of crystallisation resulted in a great decrease in the solubility.

In a study of the solubility of sheep adult and foetal Hb, Karvonen (3) came to the conclusion that the above explanation could not be the only cause of this »anomalous» type of solubility curve. In the following, the rôle of two additional factors is discussed.

Salting-out of Dilute Hb Solutions. — When dilute solutions of sheep adult or foetal Hb were precipitated by identical concentrations of ammonium sulphate, in a medium buffered with ammonium phosphates, and the total concentration of protein was varied below 1 per cent, it was observed that the protein was again salted out the more completely, the higher the starting protein concentration. However, the solubility curves differed from those of Wyman *et al.*, for in our curves, the first appearance of crystals did not immediately effect a precipitation of most of the protein; a greater amount of precipitate was needed for this. An example of this type of behaviour is shown in Table I.

TABLE I

THE RESULTS OF THE GRAVIMETRIC SULPHATE ANALYSIS OF 0.5 ML OF THE SOLUTION IN A SERIES, WHERE THE SOLUBILITY OF A SAMPLE OF SHEEP ADULT HAEMOGLOBIN (HB) WAS STUDIED AT *P*H 6.9, Γ/2: 7.50, BY APPROACHING THE EQUILIBRIUM FROM THE SUPERSATURATED SIDE

Total Hb Conc. g/1000 ml	Dissolved Hb Conc. g/1000 ml	Sulphate as mg $BaSO_4$
0.35	0.35	258.0
0.48	0.48	258.0
0.84	0.84	257.7
1.24	1.23	258.4
1.65	1.62	257.0
1.95	1.90	255.9
3.24	2.36	255.4
4.32	1.83	257.4
5.40	1.31	256.9
6.35	1.03	257.1
7.98	0.55	255.4
8.73	0.34	257.4

It was reasoned that the explanation given by Sørensen was not applicable to this type of solubility curve., when dealing with dilute solutions. Even if all the protein is precipitated, it would retain less water than its total weight is; when the concentration of protein in the final solution is about 1 per cent, the error induced in the concentration of the mother liquor by the crystal water would be, accordingly, less than 1 per cent. In fact, also some

salt is retained within the crystals, that makes the error even smaller.

This indirect conclusion was confirmed by determining the concentration of sulphate in the mother liquor gravimetrically as $BaSO_4$, in a series where increasing amounts of sheep adult Hb were precipitated at *p*H 6.9, r/2: 7.50. The results, shown in Table I, indicate that the »anomalous» shape of the solubility curve was not ascribable to variations in the strength of the mother liquor.

The Kinetics of the Precipitation Process. — If there is previously no solid in an equilibrium with the solution, the first nucleus of precipitation will, of course, be very small. As such, it has a lattice energy relatively lower and, thus, a solubility higher than an ordered macrocrystalline precipitate. Therefore, the precipitation will actually start, when the solution is saturated as compared with the amorphous precipitate of the solute. If the crystals of the solute have a high lattice energy — as for instance protein crystals — the supersaturation of the solution, as compared with the crystalline solid, may rise to considerable figures. Thus, it is essential always to state expressly, which type of solid has been used as a »reference» in a solubility study. A supersaturation in the terms of the activity of the solute in a crystalline solid may still be an »undersaturation» either in the absence of solid or in the presence of a less ordered solid state.

When the initial nucleus formation has occurred, the subsequent precipitation has often the nature and rate of an autocatalytic reaction. This finding is—at least qualitatively — a logical consequence of the initial process of nucleus formation. When there now exists a precipitate in a contact with the solution, the precipitate will sooner or later assume its thermodynamically most stable form. If the formation of an ordered crystalline precipitate is possible under those circumstances, it will at some stage occur. This means the appearance of a new solid phase, where the activity of the solute is less than in the solid phase first formed. At the beginning of the formation of the new phase the rate of precipitation will be almost proportional to the area of the new interface, and thus the rate increases autocatalytically. Later on, when the concentration of the solute will approach that of the final saturated solution, the rate will probably fall approximately according to

an equation derived for the rate of precipitation and dissolution reactions (1).

This reasoning gives a qualitatively satisfactory explanation to results such as those of Wyman *et al.*, if variations in the strength of the mother liquor prove insufficient to effect such a solubility »equilibrium». However, for the type of result presented in Table I, where the »autocatalytic» phase of the precipitation becomes manifest only after a considerable amount of the crystalline precipitate is present, neither of these explanations evidently is satisfactory.

The Rôle of »Protective Colloids». — It was observed that when an amorphous precipitate of Hb is dissolved, a small amount of amorphous non-Hb precipitate remains, which may be thought capable of acting as a »protective colloid» when in solution. It was reasoned that the possible role of »protective colloids» may be expected to appear from a comparison of the following two equilibrations: (1) a »constant solvent» series where the equilibrium is approached from the supersaturated side, using a Hb solution as the starting material; (2) a similar series, where an amorphous precipitate of Hb is used as the starting material.

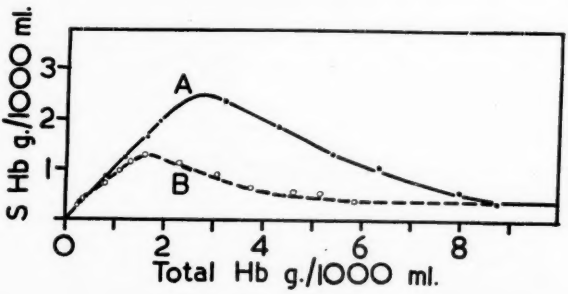


Fig. 1. — The solubility of a sample of sheep adult CO-Hb in constant solvent. *p*H 6.9, r/2: 7.50. Curve A: equilibrium approached by using a Hb solution as the starting material. Curve B: an amorphous precipitate of Hb used as the starting material. (S = solubility.)

The results of such a comparison are shown in Fig. 1. The upper diagram shows an experiment of the type (1), and the lower represents type (2). In both series, the final precipitate consisted of similar crystals and showed a complete three-dimensional order. When a great surplus of the precipitate was present, the solubility

in the two series was identical. When there was a small amount of the precipitate, a »supersaturation» was observed in both, but this phenomenon of »supersaturation» was much more pronounced in the series of the type (1).

The result of this comparison seems to indicate that the »anomalous» solubility of Hb in experiments, where the equilibrium is approached from the supersaturated side, is at least in part due to the non-Hb impurities present in the solutions. They probably act by reducing the rate at which an equilibrium is attained. Whether this is the only factor, can not be judged.

SUMMARY

In the precipitation of proteins, it is often observed that the protein is salted out the more completely, the higher the total protein concentration. The generally accepted explanation of this finding — the concentration of the mother liquor in the course of crystal water formation — is not valid, when dealing with dilute solutions. A »supersaturation» of the solution up to the solubility of an amorphous precipitate is bound to occur before the onset of the precipitation, if no crystalline solid is previously in the system. However, solubility curves were observed, where the first appearance of crystals did not immediately lead to an »autocatalytic» precipitation; a greater amount of precipitate was needed for this. A previous precipitation of the protein in an amorphous form reduced its tendency to remain in a »supersaturated» solution. This effect is ascribed to a removal of impurities capable of acting as »protective colloids» delaying a true equilibrium.

REFERENCES

1. Alexander, A. E., and Johnson, P.: Colloid Science. P. 562. Oxford: Clarendon Press, 1949.
2. Chick, H., and Martin, C. J.: Biochem. J. 1913:*7*:380.
3. Karvonen, M. J.: Thesis. Cambridge 1949.
4. Sørensen, S. P. L.: J. Amer. Chem. Soc. 1925:*47*:457.
5. Wyman, J., Raffert, J. A., and Ingalls, E. N.: J. Biol. Chem. 1944: *153*:275.

FROM THE PHYSIOLOGICAL INSTITUTE AND THE CHILDREN'S CLINIC,
UNIVERSITY OF HELSINKI

A NOTE ON THE DETERMINATION OF FRUCTOSE AND GLUCOSE BY THE SOMOGYI METHOD[1]

by

M. J. KARVONEN and O. SOMERSALO

(Received for publication June 7, 1951)

Somogyi's (6) method for determining reducing sugars in blood or in other materials has lately gained wide acceptance. It has been shown to indicate fermentable substance only, and thus to give true sugar values, whereas several other reduction methods include a variable amount of nonfermentable reducing substances in the sugar value.

According to Hitchcock (2), *fructose* and *glucose* give identical reduction values with Somogyi's method. This finding was confirmed by the present writers, when they first adopted this method. The analysis of a 10 mg per cent glucose solution gave as titration value on an average 3.36 ml, and the average titration value for an equally concentrated solution of fructose was 3.31 ml. However, when new batches of Somogyi's reagent were made, by using analytical reagents obtained from different sources, the reduction values for equally strong glucose and fructose solutions differed. This difference was found also when the analysis was performed by using the colorimetric modification of Nelson (5). The results obtained with one batch of the reagent are given as an example in the form of a table.

---

[1] A correction to M. J. Karvonen and O. Somersalo, »Comparison of Peroral Fructose and Sucrose Tolerance Tests in Normal Rabbits» (*Ann. Med. Exp. Biol. Fenniae*, Vol. 27, 1949, pp. 30—40).

A similar difference between the reduction values of fructose and glucose has been reported by Miller & Van Slyke (4), when studying the applicability of their own method. However, Bacon & Bell (1) have obtained by the same method identical reduction values with these two sugars.

It is evident that the applicability of at least these two reduction methods depends essentially on the source of the analytical reagents or on some other uncontrolled factor.

In order to study the cause of these variations in the Somogyi reaction, the oxidation reagent was made up in 14 combinations, by using reagents produced by a number of manufacturers. Three different samples of KNa-tartrate, five samples of $CuSO_4$, and five samples of Na_2SO_4 were emplyed in preparing the series. The reagents thus made were tested on 10 mg per cent solutions of glucose and fructose, respectively, altogether four times during a period of three months.

The results showed:

a) that the different combinations all gave almost identical blank values with distilled water;

b) that at the first determination after making the solutions, the reduction value of glucose varied between 96 and 106 per cent of that of fructose;

c) that the initial reduction values varied slightly with the different reagents used;

d) that on keeping the solutions at room temperature, the reduction values of both sugars fell; the reduction value of glucose decreased much more than that of fructose, down to 15 per cent of its original value, and to 27 per cent of the simultaneous reduction value for fructose;

e) that great variations occurred in the rate of the deterioration of the different combinations of Somogyi's reagent;

f) that the source of the $CuSO_4$ seemed to be the most important factor in determining the stability of the Somogyi reagent.

The extensive use of these methods in medical and other laboratories makes it desirable that the marketing firms should produce such analytical reagents as have specially been tested for their applicability to the sugar reduction methods.

TABLE

A Comparison of Fructose and Glucose as Standards of the Somogyi Method

	Fructose	Glucose	Glucose per cent of the Fructose Value
Titrimetric:	*Ml*	*Ml*	
10 mg per cent solution	3.48	2.98	86
5 » » » »	1.90	1.40	74
1 » » » »	0.53	0.47	89
Photometric:	*Reading*	*Reading*	
10 mg per cent solution	0.161	0.110	68
5 » » » »	0.093	0.069	74
1 » » » »	0.021	0.015	71

The present findings affect some of the results presented in a paper by Karvonen & Somersalo (3). In this work, some of the batches of Somogyi's reagent were standardised only by using fructose. Later results have made it evident that this led to a methodical error: to too low blood sugar values. The fall of the basal sugar values reported on p. 36 of the paper depends therefore on a methodical error. An attempt to repeat the result by similar technique failed. The values presented in Table IV (p. 37) are also subject to a correction. However, these methodical errors do not invalidate the main conclusion of the study: the equivalence of fructose and sucrose in tolerance tests.

SUMMARY

Depending on the source of reagents, Somogyi's method may give either identical or greatly different reduction values for fructose and glucose. On keeping the reagent tends to deteriorate; in particular, it loses its ability to oxidize glucose. On the basis of this observation, some of the findings published by Karvonen and Somersalo (3) are corrected.

REFERENCES

1. Bacon, J. S. D., and Bell, D. J.: Biochem. J. 1948:*42*:397.
2. Hitchcock, M. W. S.: J. Physiol. 1949:*108*:117.
3. Karvonen, M. J., and Somersalo, O.: Ann. Med. Exp. Biol. Fenniae (Helsinki) 1949:*27*:30.
4. Miller, B. F., and van Slyke, D. D.: J. Biol. Chem. 1936:*114*:583.
5. Nelson, N.: J. Biol. Chem. 1944:*153*:375.
6. Somogyi, M.: J. Biol. Chem. 1945:*160*:61.

FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

EFFECT OF WELCHI TOXIN ON HUMAN HEPATITIS SERA

by

INGA RUDBERG-ROOS

(Received for publication August 15, 1951)

»The Turbidity-producing Action of *Clostridium perfringens* Toxin in Human Sera» was the title of an academic dissertation published by M. Tuomioja (22) in Helsinki at the beginning of 1950. Referring to earlier investigations (12, 19), Tuomioja called this phenomenon the Seiffert-Nagler Reaction, abbreviated to SNR, a name which will be used in this paper too. It was shown by Macfarlane *et al.* (10) in 1941 that similar turbidity was produced in a suspension of egg yolk in saline. In 1947 Renkonen (16) showed that the reaction was more rapid in hepatitis sera than in normal sera and gave results that are comparable with and corresponding to the thymol turbidity test. Tuomioja's study was an additional investigation of the reaction on the basis of this observation.

Tuomioja's method was as follows:

0.5 ml of inactivated serum, 0.5 ml of physiological saline solution and 0.5 ml of toxin (passed through a Seitz filter) were mixed. After shaking the estimations were performed in a Zeiss nephelometer. Estimations were made continuously every 15th minute, while the test tubes in the meantime were placed in a water bath at 37° C. A mixture of 10—20 different normal sera was used as the standard. — Parallel with this serum-SNR Tuomioja also performed to a great extent a SNR of serum and egg yolk, i.e. serum + toxin + 0.5 ml of a suspension of egg yolk in saline (one egg yolk was mixed in 250 ml of saline and passed through a Seitz filter). Every test was recorded and a curve was drawn with the time on the abscissa and the nephelometrical value on the ordinate. The time required for the mixture of normal sera to reach a turbidity increase of 200 (the

initial value = 0) was made the value 0, around which the other sera were grouped as positive and negative values. According to the graduation + 10 is the highest possible value, i.e., for a serum which is turbidified immediately. — For comparison he performed thymol turbidity tests (henceforward abbreviated TTT); these were performed with inactivated serum.

Tuomioja's investigation shows that after a lag phase of varying length turbidity follows and reaches its maximum after a period of various length. The length of the lag phase is inversely proportional to the amount of toxin used and is prolonged when the amounts of serum are increased. Haemolytic sera show a considerably retarded reaction. The variations of time when reactions are carried out with active or inactivated serum are slight. Bile acids (0.03 per cent sodium taurocholate and glycocholate) effect an apparent retardation of the reaction. Added α-globulin gives a prolonged lag phase, while albumin only makes the slope of the curve steeper. — The results of his clinical investigations may be summarized as follows: the SNR is positive in hepatitis (27 cases of 36) being as a rule maximal at the beginning of the disease, then it rapidly decreases and is thereby distinguished from the TTT which shows increasing values during the course of the disease and a slower decline. Furthermore, new-born infants have positive SNR values (umbilical cord blood), while the TTT are quite negative. Negative SNR values were found in late pregnancy, in puerperium and in cases of certain tumours, especially malignant ones.

As far as I have been able to find, it is of importance, whether the reaction is performed with active or inactivated serum. See Table I. When active serum is used the reaction in cases of »slow» sera, i.e. sera with negative SNR, is still more retarded. When »rapid» sera, i.e. sera with positive SNR, are concerned, the tendency is more variable. Schmidt (18), who has studied the effect of welchi toxin on active serum, is of the opinion that inactivation is without importance for producing turbidity.

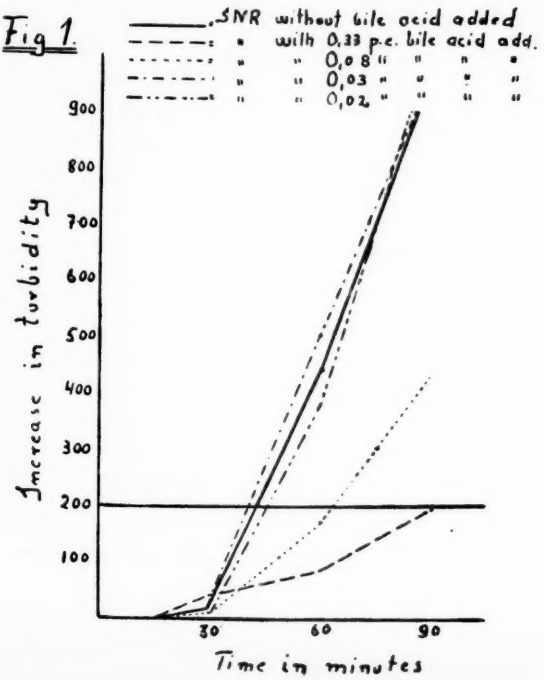
The addition of bile acids (sodium taurocholate) in concentrations of 20—30 mg per 100 cc shows no effect on the course of the reaction, neither with normal serum nor with hepatitis serum. Not until the concentrations were 80 mg per 100 cc or more did there appear a distinct retardation (See Fig. 1). According to Everett (6)

TABLE I

No.	Diagnosis	SNR (Inact.)	SNR (Act.)
1.	Hepatitis	+6	+8
2.	»	—4	—15
3.	»	0	—1
4.	»	+3	+2
5.	»	—3	—12
6.	»	+2	+3
7.	»	+6	+6
8.	»	+7	+8
9.	»	+1	—6
10.	»	+5	+6
11.	»	0	0
12.	»	0	—6
13.	Biliary cirrhosis	+7	+10
14.	» »	+9	+10
15.	Pulmonary cancer	+3	0
16.	» »	—3	—2
17.	Rectal cancer	—4	—6
18.	Cholecystitis	—1	—6
19.	»	+1	—1
20.	Normal serum	0	—4

Nos 10—12 are samples from the same patient with intervals of a fortnight.

Fig 1.



the concentration of bile acids in the blood *may* amount to 30 mg per 100 cc in cases of obstructive jaundice and to 10 mg per 100 cc in hepatitis. Thus, no effect of the amounts that can exist in diseases is seen *in vitro*. This is in accordance with the results of Macfarlane and Knight (9) who after the addition of sodium taurocholate, 0.08 per cent, found that the activity of the enzyme had been reduced by 10 per cent.

On one occasion, when the temperature in the refrigerator went down below 0° C, a hepatitis serum froze totally, while the other sera, that were not from hepatitis cases, remained unchanged. On account of this observation, tests were made with both active and inactivated hepatitis sera that were placed in a cold-storage room with a temperature of —5° C. The results were extremely divergent, the »freezing time» ranging from 1 hour to 4 days; as to active and inactivated serum from the same patient it varied from 1 to 50 hours. Throughout, the »freezing time» of the active serum was shorter. Any connection on one hand with the time, on the other hand with the SNR- and TTT-values could not be proved. The tests with normal sera and sera from patients (not hepatitis cases) gave equally varying results. Active sera did not show any tendency to give a shorter »freezing time».

It is known that hepatitis sera have a tendency to produce non-specific serological reactions, so for instance, in antistreptolysin titre (= AST.) determinations. According to Oker-Blom *et al.* (13) this non-specific AST. can be eliminated through lipid extraction with acetone and ether (3). His attempt to get the same result with bentonite (7) does not, however, in concentrations of 0.20 per cent (serum dilution 1: 10) show any significant decrease of the »hepatitis-AST.» though the decrease of lipids was approximately the same as that produced by the extraction with acetone and ether, i.e. about 75 per cent both in case of cholesterol and of phospholipids. — In order to see how the SNR would turn out with sera from which the lipids had been extracted, the test was carried out after bentonite adsorption (13). A distinct retardation of the reaction seemed to set in with bentonite concentrations of about 1 per cent (undiluted serum), but also with a 2 per cent solution, turbidity appeared little by little (after 40—100 hours in contrast to 1 hour in non adsorbed serum) (See Fig. 2). Determinations of lipids before and after adsorption gave the same results as

Fig 2.

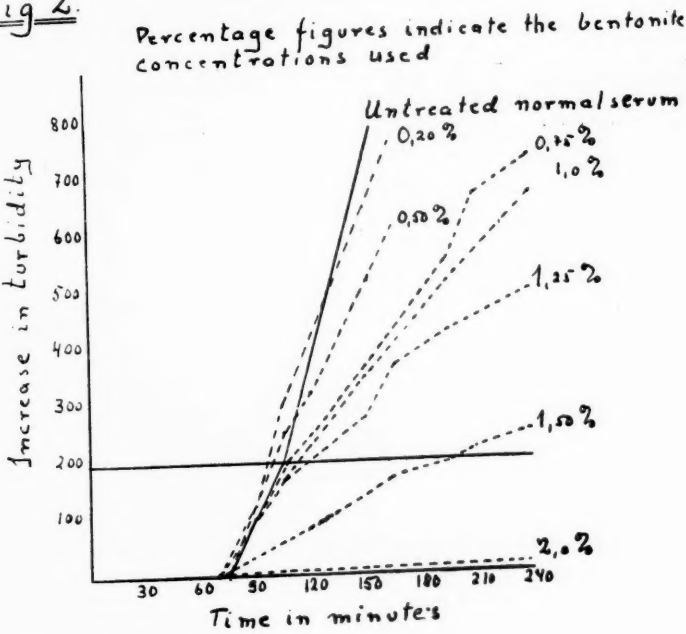

Percentage figures indicate the bentonite concentrations used
Untreated normal serum
0,20 %
0,25 %
1,0 %
0,50 %
1,25 %
1,50 %
2,0 %
Increase in turbidity
800
700
600
500
400
300
200
100
30
60
90
120
150
180
210
240
Time in minutes

Fig 3.

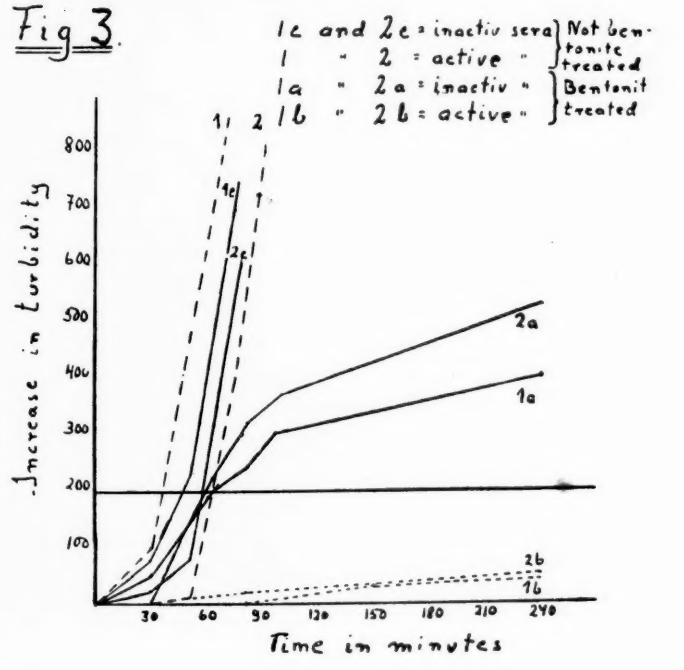

1c and 2c = inactiv sera } Not bentonite treated
1 " 2 = active " }
1a " 2a = inactiv " } Bentonit treated
1b " 2b = active "
Increase in turbidity
800
700
600
500
400
300
200
100
1c
2c
2a
1a
2b
1b
30
60
90
120
150
180
210
240
Time in minutes

TABLE II

TTT WITH SERUM TREATED WITH BENTONITE

Untreated Serum		Serum Treated with Bentonite					
Active	Inactive.	Active 1 per cent	Inactive. 1 per cent	Active 1.5 per cent	Inactive. 1.5 per cent	Active 2 per cent	Inactive. 2 per cent
7.8	6.2	4.4	5.2	2.2	3.6	0.6	1.8

those in Oker-Blom's investigation (13). Tests with both active and inactivated bentonite serum showed distinctly that the retardation was more pronounced with active sera than with inactivated ones (See Fig. 3). For comparison, a number of TTT were also performed with active and inactivated sera treated with bentonite. A typical series of tests is shown in Table II. Here, too, a distinct reduction of the values is produced by the 1 per cent bentonite solution. The reduction is greater in active serum than in inactivated one, although the TTT gives higher values with active serum. See also Brante (5). Recant *et al.* (15) have demonstrated previously that the thymol reaction becomes negative after extraction of lipids with ether in the cold.

CLINICAL INVESTIGATION

After rejecting 55 turbid or haemolytic samples, the material consisted of 201 cases, 59 being cases of hepatitis.[1] Of these cases, 34 were examined twice or several times. Altogether there were 134 determinations. Only those cases which, according to the hospital records, were diagnosed as certain cases of hepatitis, have been included. According to Tuomioja the value of normal sera (211 samples) ranged from +4 to —1. I have examined only 45 cases, the values of which showed great deviations, from +3 to —4, the average value being —0.9. I have considered the values between +2 and —3 as normal ones.

The distribution of the results is indicated by Fig. 4. There is a distinct tendency to positive values among the hepatitis cases, although a number of values lie over on the negative side. Fig. 5 shows the values of the hepatitis cases at the admission and at

[1] The hepatitis sera have with few exceptions come from the Hospital for Epidemic Diseases, the other sera from the Medical Departments of the Kivelä and Maria Hospitals. The investigation was begun in November 1949 and continued until June 1950.

Fig 4

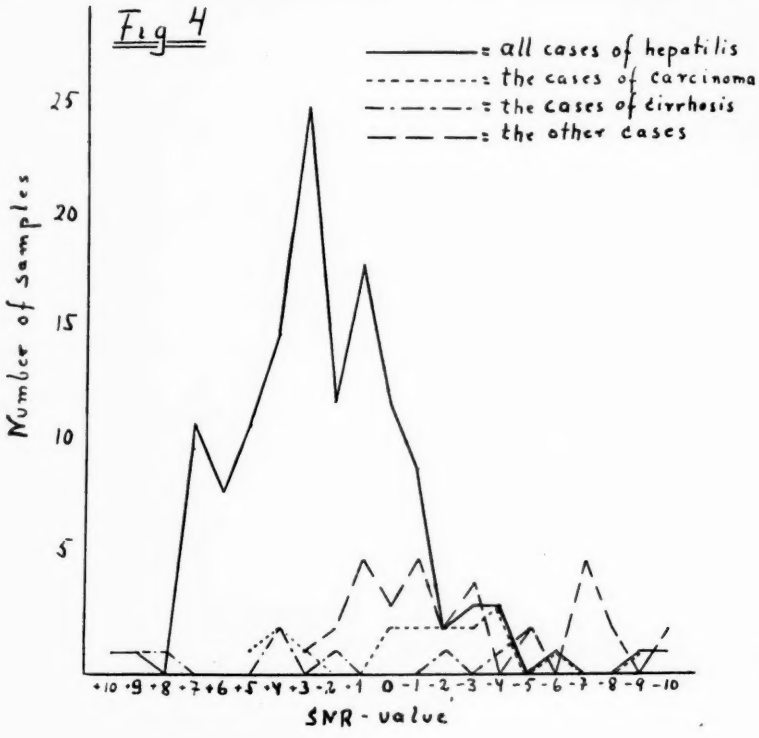

= all cases of hepatitis
= the cases of carcinoma
= the cases of cirrhosis
= the other cases
Number of samples
25
20
15
10
5
+10 +9 +8 +7 +6 +5 +4 +3 +2 +1 0 -1 -2 -3 -4 -5 -6 -7 -8 -9 -10
SNR - value

Fig 5

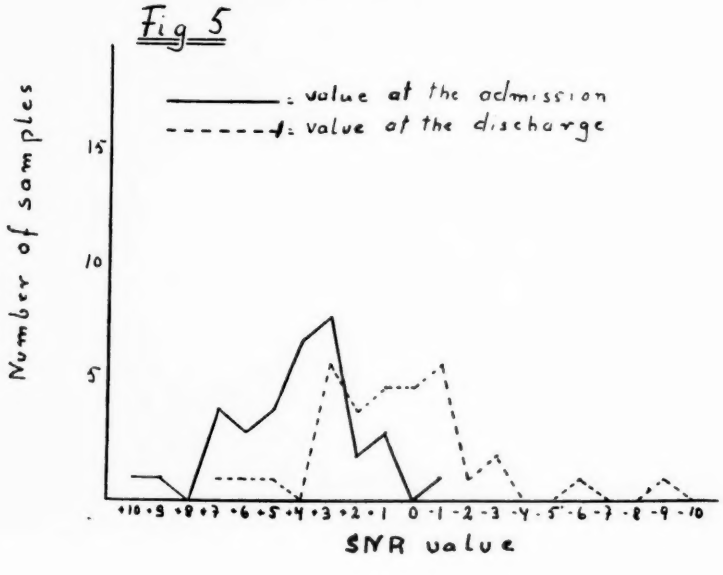

= value at the admission
= value at the discharge
Number of samples
15
10
5
+10 +9 +8 +7 +6 +5 +4 +3 +2 +1 0 -1 -2 -3 -4 -5 -6 -7 -8 -9 -10
SNR value

TABLE III

F. = FEMALE. M. = MALE. TH. = TTT

No.	Sex	Onset of Disease	Date of Admission	SNR and TTT 1	2	3	4	5
1.	F.	15.11.	21.11.	23.11. +9	29.11. +7,Th.10	5.12. +1,Th.6	16.12. +3,Th.4	
2.	F.	29.9.	15.10.	24.11. +3	5.12. —10,Th.4	17.12. —3,Th.4	29.12. —3,Th.4	
3.	M.	1.11.	23.11.	24.11. +6	5.12. +3			
4.	F.	19.10.	8.11.	29.11. +5,Th.8	9.12. 0,Th.4			
5.	M.		26.11.	29.11. +7,Th.8	16.1. +7,Th.3	27.1 +1,Th.2		
6.	F.	12.11.	16.11.	5.12. +4,Th.2	17.12. +2,Th.1			
7.	F.	3.12.	9.12.	16.12. +3.	29.12. +1,Th.4			
8.	M.	1.12.	14.12.	17.12. +1,Th.3	27.12. +2,Th.4	16.1. 0,Th.2	27.1 —4,Th.2	1.2. +3,Th.3
9.	F.	15.12.	22.12.	27.12. +1,Th.12	3.1. —9,Th.5			
10.	F.	1.12.	14.12.	27.12. 0,Th.7	2.1. +5,Th.8	14.2. +7,Th.8	23.2. 0,Th.8.	
11.	M.	1.12.	21.12.	27.12. +4,Th.5	2.1. +7,Th.7	19.1. —1,Th.3	27.1 —1,Th.3	
12.	M.	4. 1.	14. 1.	16.1. +4,Th.14	19.1. +1,Th.12	31.1. +3,Th.14	6.2. +3,Th.11	11.2 +3,Th.13
13.	M.	9. 1.	12. 1.	18.1. +4,Th.2.	27.1. +4	1.2. +4,Th.2	7.2. +1,Th.1	11.2. +3,Th.2
14.	M.	17.1.	18.1.	27.1. +5,Th.12	30.1. +6,Th.14	6.2. +5,Th.8		
15.	F.		31. 1.	2.2. +3,Th.13	7.2. +2,Th.8	13.2. +2,Th.8		
16.	F.	1. 2.	8. 2.	9.2. +7,Th.9	14.2. +2,Th.10	23.2. +3,Th.8		
17.	F.	4. 2.	11. 2.	14.2. +6,Th.15	23.2. +6,Th.20	11.3. 0,Th.13		
18.	M.	15. 2.	15. 2.	25.2. +10,Th.6	7.3. +7,Th.6	20.3. +3,Th.5	24.3. 0,Th.4	29.3. +2,Th.3

TABLE III (Contin.)

No.	Sex	Onset of Disease	Date of Admission	SNR and TTT 1	2	3	4	5
19.	F.	19. 2.	22. 2.	23.2. +3,Th.12	2.3. +4,Th.6	6.3. +4,Th.8	17.3. +2,Th.8	27.3. 0,Th.6
20.	F.	24. 2.	27. 2.	10.3. +2,Th.6	20.3. —1,Th.4	27.3. 0,Th.4		
21.	F.	7. 3.	14. 3.	15.3. +3,Th.17	27.3. +1,Th.16			
22.	M.	5. 3.	23. 3.	28.3. +4,Th.5	3.4. +5,Th.5	11.4. —1,Th.4		
23.	F.	8.3.	28. 3.	29.3. +1,Th.12	18.4. —1,Th.6			
24.	M.	2. 4.	6. 4.	17.4. +4,Th.17	24.4. +3,Th.14	2.5. —2,Th.12		
25.	M.	11. 4.	14. 4.	17.4. +3,Th.6	26.4. +3,Th.5			
26.	F.			9.5. +5,Th.5	17.5. +5,Th.6	30.5. +2,Th.2		
27.	F.	9. 5.	11. 5.	12.5. +7,Th9.	22.5. +6,Th.7			
28.	F.	13. 5.	15. 5.	16.5. +1,Th.8	26.5. —3,Th.5			
29.	F.	23. 4.	16. 5.	17.5. +5,Th.6	26.5. 0,Th.3	5.6. —1,Th.3	14.6. 0,Th.2	
30.	F.	8. 5.	16.5.	19.5. +7,Th.18	30.5. 0,Th.14			
31.	F.	25. 5.	25. 5.	26.5. +3,Th.7	5.6. +8,Th.6	21.6. +1,Th.7		
32.	M.	17. 5.	25. 5.	5.6. +3,Th.1	14.6. +1,Th.2			
33.	M.	28. 5.	10. 6.	14.6. +7,Th.9	26.6. +7,Th.13			
34.	F.	4. 6.	14. 6.	14.6. +6,Th.15	22.6. —1,Th.11			

the discharge from the hospital, and it is to be seen that they have been normalized during the course of the disease. The values of the 34 cases of hepatitis which have been examined more than once are shown in Table III. The typical course of disease as described

by Tuomioja with a high SNR at the admission, which rapidly declines, is distinctly to be found in 11 cases of the 34 examined. Even pronouncedly negative values exist during the regress of the disease. It seems more usual that the variation of the values in one and the same patient is not very great. SNR values that reach the maximum later on during disease, or high values that persistently remain, are also to be noted. In 3 cases the TTT is positive, while the SNR shows normal values. Further, 4 cases have negative TTT but a slightly or moderately increased SNR (incorrect diagnosis?). — According to the table the SNR is positive in 30 cases of 34). As to the TTT the result is identical. (POSITIVE in 30 cases of 34). The cases number 2 and 7 both have a TTT value of 4, but as this determination was made late during the course of the disease, one may suppose that the value had been higher previously. It is possible that the ordinary upper normal value of the TTT, +4, which is often considered too high (20), would more likely correspond to the SNR +3 than to +2. In that case the observations will give only 23 positive cases of 34, quite an important difference.

In the cases of cirrhosis, especially the variation of the values is striking as well as the lack of correlation in the results between the SNR and the TTT. See Table IV. In my material, I have only 7 cases of bile duct affections, the values varying between +3 (1 case) and —5. Tuomioja had 11 cases (7 cases of cholecystitis with the SNR ranging from +3 to —2, 3 cases of cholelithiasis (+1), and 1 case of choledocholithiasis (—1). Accordingly, no

TABLE IV

SNR AND TTT IN CASES OF CIRRHOSIS

No.	Diagnosis	SNR	TTT
1.	Cirrhosis (Laënnec)	—5	12
2.	» »	—5	6
3.	» »	—2	9
4.	» »	+4	20
5.	» »	—4	14
6.	» »	+2	8
7.	Biliary cirrhosis	+4	8
8.	» »	+9	1
9.	» »	+8	3

signs indicating a positive SNR in obstructive jaundice have so far been obtained. The cases of cancer show throughout low values though not lower ones than the cases of cardiac insufficiencies. The investigation also consisted of 21 samples from average medical material. All values were normal or slightly negative with the exception of a case of myeloma and a case of seropositive lues, both of which had a markedly negative SNR and a highly positive TTT (20 and 10). Finally, 5 samples from new-born infants (umbilical cord blood) showed pronouncedly negative values. The samples were not haemolytic, and I have not been able to find any explanation for this result which is wholly divergent from Tuomioja's findings.

As far as I have been able to find, the reaction includes many uncertain factors, which will partly suffice to explain the discrepancy between the results of Tuomioja and myself. In the case of the toxin the strength has varied considerably in different preparations. It is obvious, as has also been pointed out previously (16), that a very strong toxin is unsuitable, as it gives too small a variation of the lag phase. Otherwise, the varying strength of the toxin will hardly be of any importance. The risk of bacterial contamination has not been insignificant, but it has been eliminated through freezing (—20° C). On the other hand merthiolate was directly unsuitable as a preservative, because it produced an extreme retardation of the reaction (concentration 1: 5000).

The mixtures of normal sera, which make the »standard» of the reaction, show unfortunately rather great variations from one another. The passing through a Seitz filter of the mixtures effected sometimes an accelerated reaction, sometimes the contrary, and it was thus unusable as an attempt to prevent contamination, the risk of which, however, did not seem to be great.

To begin with, tests were carried out both with the »serum-SNR» and the »egg yolk-SNR». The latter was then wholly rejected, because the egg yolk suspension was not very durable. Tuomioja's reasons for its use, among other things the elimination of variations in the curves of different sera, seem to me a disadvantage.

Finally, as to sera from patients, I got the impression that hepatitis sera would not be as well preserved as other sera. After 3 days in the refrigerator they sometimes showed small flocculent

precipitates, though without signs of bacterial contamination. These sera were not used for testing.

TTT have been carried out parallel with the SNR, but using active instead of inactivated serum, because the inactivation produced a reduction of the values and this reduction was not uniform.

What is Known about the Reaction? — The lecithinase in the welchi toxin splits the lecithin into phosphocholin and diglyceride according to Macfarlane and Knight (9). Ahrens and Kunkel (1) demonstrated in 1949, that clear sera with a high lipid percentage existed only in obstructive jaundice, with a high concentration of phospholipids. Turbid sera could not be clarified through the addition of phospholipids, but clear sera could be rendered turbid with lecithinase (welchi toxin). The sera were turbidified after a certain lag phase, but already during this period the lipid phosphorus was reduced and the acid-soluble phosphorus increased. A similar opinion about the course of the reaction has been expressed previously (9). The values these authors arrived at showed also, that the total quantity of cholesterol is lower and that the percentage of free cholesterol is higher throughout in clear sera than in turbid sera. Equally, the concentration of neutral fat is lower in clear sera than in turbid sera. As a further proof of the importance of the phospholipids, a case of hepatitis, reported by Boyd and Connell (4), is mentioned. It showed a low total percentage of lipids and a very low concentration of phospholipids with spontaneous turbidity in the serum. The authors made a reservation, however, concerning the effect that bile acids may have on the clearness of lipaemic sera.

Can the positive SNR be explained exclusively by the reduced quantity of phospholipids in the serum? Certainly not. In my material, only one case of hepatitis and one case of biliary cirrhosis as well as two normal sera, have been lipidexamined. The former ones had a highly positive SNR. The lipid phosphorus values were increased, 30 mg per 100 cc and 60 mg per 100 cc, while the values of the normal sera were approximately 10 mg per 100 cc. Lipid determinations in normal sera and in hepatitis sera are listed in Table V. Table VI shows 3 cases of hepatitis examined serially.

The cases of cirrhosis show total values of cholesterol that are round about the lower normal value (2, 11, 17, 21) specially in cases

TABLE V

Authority	Number of Cases	Total Lipids	Neutr. Fat	Fatty Acids	Phospholipids	Total Chol.	Free Chol.	Free: Total Chol.
Normal values given in mg per 100 cc unless stated otherwise.								
Ahrens & Kunkel	25	636±171			219±55	185±48	56±15	0.30±0.09
Albrink	23				220 [1]			
Boyd & Connell	—	617±75	154	362	195±37	181	53	
Peters & Man ..	100			12.3 (m.Eq./l.)	230±35 (153—363)	194±36 (107—320)		0.26—0.28
Cases of hepatitis								
Albrink	6			19.1 (m.Eq./l.)	338 [1]	212	107	
Man *et al.*	17				150—450	80—310		
(after recovery)					(225—300)	(150—280)		
Oker-Blom *et al.*	2				369	78		
					536	178		
Tayeau *et al.* ..	5	1024 (441—1264)			195 (123—277)	210 (123—277)		

[1] The value is given in mg per cent lipid phosphorus, transformed to phospholipids after multiplication with 25 (18).

TABLE VI

Author	Neutr. Fat (m.Eq./l.)	Fatty Ac. (m.Eq./l.)	Phospho-lip. mg/100 cc	Chol mg/100 cc	Free Chol. mg/100 cc	Day during Course of Disease
Albrink		18.5	348	159	113	1
		21.1	433	242	144	8
		16.3	308	230	106	12
		12.3	252	242	67	39
Man *et al.*	13		375	160	115	1
	7		350	320	120	24
	7		275	275	100	117
Man *et al.*	8		250	130	105	
	6		188	150	50	—
	7		213	140	50	

of simultaneous ascites (2). The same applies to the phospholipids. In cases of biliary cirrhosis, biliary occlusion and primary liver cancer (11, 17, 21) both cholesterol and phospholipids are considerably increased.

The extensive normal material (Table V) of Peters and Man (14) shows that the range of variations is very great. Man *et al.* (11) lay stress upon the great variations that are seen in cases of hepatitis emphasizing the fact that there are 2 types of hepatitis, one in patients with acholic faeces with a moderate hyperlipaemia (a moderate increase of cholesterol and phospholipids and a somewhat greater increase of neutral fat). The other type shows hypolipaemia, which may be of serious prognostic import. As a rule, the cases of toxic hepatitis belong to this type. The only constant change in cases of hepatitis seems to be the considerably reduced cholesterol esters. It seems as if these were reduced approximately during the same time as the SNR is positive (3 cases!) A serial fractionation of lipids parallel with the determination of the SNR has unfortunately been impossible to carry out. It is possible that such fractionating might give results that could correlated with the course of the SNR curve. Any conclusions concerning quantitative lipid changes as a cause of a positive SNR cannot be drawn as yet.

According to Tayeau *et al.* (21) in cases of jaundice the serum-lipids can be extracted to a great extent, simply by shaking with ether, which is not the case with normal sera. The writers suppose that this occurs owing to the adsorption of the bile salts to the proteins, by which the lipids are »repelled» and thus are easily liberated. The serum from patients with haemolytic jaundice reacts like normal serum. The investigation indicates that 2—6 per cent of cholesterol and 1 per cent of phospholipids can be extracted from normal sera, while the corresponding figures for 10 cases of obstructive jaundice and 5 cases of hepatitis reached 99 per cent and 40—60 per cent respectively. — A similar observation has been made by Longsworth *et al.* (8): A large part of the high β-globulin in the serum from cases of obstructive jaundice disappears after shaking with ether.

Reubi (17) who has also examined the ether extraction in liver diseases, found an increased extraction of cholesterol (about 45 per cent) in cases of hepatitis and often in cases of cirrhosis. An increased extraction of fatty acids and phospholipids, too, was found

in cases of biliary cirrhosis and those of choledochus cancer but also in a case of primary liver cancer without jaundice. He rejects the bile acid theory and presumes that the increased extraction depends on a disturbance in the lipids, which results in altered affinity for the proteins.

The SNR does not give positive values in cases of obstructive jaundice, which according to Tayeau *et al.* (21) shows conditions of extraction identical with the cases of hepatitis. However, the observation is worth noting and deserves closer investigation.

DISCUSSION

The SNR is positive in most cases of hepatitis, i.e. in approximately 80 per cent of the cases. If, considering the sources of error in the method, the demands for positive reactions are increased, the positive tests amount to 65—70 per cent, which figure will for certain be increased with an improved technique. Accordingly, the reaction gives results inferior to the TTT, and it is far too time-consuming to be suitable for clinical use. A simplification of the method might be carried out as follows: Estimation with the naked eye 2—4 times with intervals wholly depending on the strength of the toxin ought to give, after some practice, values that are exact enough.

CONCLUSION

1) Welchi toxin mixed with serum produces turbidity, which appears more rapidly in hepatitis sera than in other sera.

2) In its present form the reaction is too time-consuming to be suitable for clinical use. Besides, it gives results inferior to the TTT.

3) A simplification of the test is suggested.

4) The reaction is of great theoretical interest, but the mechanism is still on the whole unknown.

REFERENCES

1. Ahrens, E. H., and Kunkel, H. G.: J. Exper. Med. 1949:*90:*409.
2. Albrink, M. J.: J. Clin. Invest. 1950:*29:*46.
3. Blix, G.: J. Biol. Chem. 1941:*137:*495.
4. Boyd, E. M., and Connell, W. F.: Arch. Int. Med. 1938:*61:*755.
5. Brante, G.: Sv. Läkartidn. 1946:*43:*2661.
6. Everett, M. R.: Medical Biochemistry, 1948:198.
7. Hansen, A.: Acta Path. et Microbiol. Scand. 1948:*25:*460.
8. Longsworth, L. G., Shedlovsky, T., and MacInnes, D. A.: J. Exp. Med. 1939:*70:*399.
9. MacFarlane, M. G., and Knight, B. C. J. G.: Biochem. J. 1941:*35:* 884.
10. MacFarlane, M. G., Oakley, C. L., and Anderson, C. G.: J. Path. Bact. 1941:*52:*99.
11. Man, E., Kartin, B., Durlacker, S., and Peters, J.: J. Clin. Invest. 1945:*24:*623.
12. Nagler, F. P. O.: Brit. J. Exp. Path. 1939:*20:*473.
13. Oker-Blom, N., Nikkilä, E., and Kalaja, T.: Ann. Med. Exp. Biol. Fenn. 1950:*28:*125.
14. Peters, J. P., and Man, E. B.: J. Clin. Invest. 1943:*22:*707.
15. Recant, L., Chargaff, E., and Hanger, F. M.: Proc. Soc. Exp. Biol. & Med. 1945:*60:*245.
16. Renkonen, K. O.: Ann. Med. Exp. Biol. Fenn. 1947:*25:*155.
17. Reubi, F.: Helvet. Med. Act. 1948:*15:*262.
18. Schmidt, H.: Z. Immunit. forsch. 1941:*100:*241.
19. Seiffert, G.: Z. Immunit. forsch. 1939:*96:*515.
20. Tallroth, A.: Diss. Uppsala 1949.
21. Tayeau, F., Rolland, R., and Blanquet, P.: J. Méd. Bord. 1948: 125/10:433.
22. Tuomioja, M.: Ann. Med. Exp. Biol. Fenn. 1950:*28:*Suppl.2.

FROM THE DEPARTMENTS OF MEDICAL CHEMISTRY AND OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

EFFECT OF TREATMENT WITH BENTONITE ON SERUM PROTEIN, LIPID PHOSPHORUS, AND CHOLESTEROL

by

ESKO NIKKILÄ and NILS OKER-BLOM

(Received for publication August 17, 1951)

In a previous paper certain results connected with the non-specific antistreptolysin reactions (AST) occurring in sera of patients with epidemic hepatitis have been presented (12). It was assumed that the non-specific AST reactions were due to an interaction between the serum lipids and the streptolysin O, and that the real AST value of these sera could be obtained if the serum lipids were removed. To some extent this reasoning seems valid, as has also been shown by Packalen (16) earlier.

The removal of serum lipids, however, without changing the serum proteins, is difficult. Plain ether extraction at room temperature does not remove the lipids. This is possible only after dehydration with cold alcohol (6) or acetone (1), or if the serum is chilled to —25° C (9). Thus all these methods require low temperatures, and are therefore laborious for a serological laboratory. Kunkel *et al.* (8) state that the dilution of a serum with phenol of high salt concentration precipitates the lipids without affecting the serum proteins, but they give no accurate values. Finally, betonite (montmorillonite) has been used by Hansen (5) for the removal of antiproteolytic serum factors, *i.a.* lipids for the purification of diphtheria antitoxin. It was therefore assumed that the lipid extraction at cold temperatures could probably be replaced by absorption of the lipids to bentonite, which can be performed at room temperature.

Some chemical analyses performed on fresh and bentonite treated sera showed that the main part of the lipids were removed without appreciably changing the protein content of the sera, and according to electrophoretic analyses of the same sera the serum fractions mostly affected were the α- and β-globulins, where the greatest part of the serum lipids are supposed to occur. It is reasonable to assume that the change in the electrophoretic pattern of these serum fractions is due to the removal of the lipoprotein complexes, but as long as we do not know the exact composition of the different globulin fractions it may also be assumed that probably other components of these globulin fractions are removed by the bentonite treatment. From a serological point of view this was of considerable interest, and certain other serological experiments were performed.

The agglutination of living hemolytic streptococci by sera of patients suffering from rheumatoid arthritis has long puzzled the serologists (10, 2, 3, 4, 7, 15, 22). In recent years this agglutination reaction has been ascribed to some non-specific factor(s) in the sera (20, 11, 13, 19, 17). It was assumed that some such non-specific serum factor could probably occur in the α- or β-globulin fraction of the sera, and accordingly be removed with bentonite (14). This also seemed to be the case, in that the ability of the rheumatoid arthritis sera to agglutinate living hemolytic streptococci disappeared completely after bentonite treatment, whereas the bentonite treatment did not affect the agglutination of *S. typhi* and *S. paratyphi B* and *B. abortus Bang* or *Proteus X 19* in specific immune sera or in patients' sera, no more than the isoagglutination of human red cells in blood donors' sera.

Since bentonite thus removes the serum lipids, and probably also other serologically active serum components, it was considered interesting to obtain a closer knowledge of the true effect of bentonite on human sera, and this paper deals with the chemical and electrophoretic analyses of sera treated with bentonite.

MATERIAL AND METHODS

Serum. — Fresh blood donors' sera have been used. Five sera were tested in all.

Bentonite. — The bentonite preparation is produced by H.

Struer's chemical laboratory, København and has kindly been placed to our disposal by Dr. A. Hansen, State Serum Institute, Copenhagen. The bentonite suspensions were made up in distilled water.

Chemical Analyses. — The proteins were determined by the micro Kjeldahl method. The lipid determinations were performed using a method described by Vesa and Kalaja.

Electrophoretic Analyses. — The electrophoretic analyses were performed with the apparatus described by Tiselius and using the optic system described by Svensson. The serum was subjected to dialysis against a phosphate NaCl buffer (*p*H 7.8; ionic strength 0.15) for three days and diluted with the same buffer to a final protein concentration of 1.5 per cent. The electrophoresis was carried out at a temperature of + 1.0° C with a potential gradient of 5—6 volt per cm, and for 135 minutes.

EXPERIMENTAL

Effect of Bentonite Treatment on Serum Protein, Lipid Phosphorus and Cholesterol at Different pH of the Serum. — Using 0.1 n hydrochloric acid and 0.1 n sodium hydroxide solutions, the *p*H of aliquots of the same serum diluted one to one was adjusted to 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. To each aliquot of serum an equal part of an 0.4 per cent bentonite suspension was added and the tube shaken gently. Thus the final bentonite concentration of the serum bentonite mixture was 0.2 per cent. The mixture was kept at room temperature for 20 minutes to half an hour and centrifuged at 1,500 rpm for ten minutes. From the supernatant the serum protein, the lipid phosphorus and the serum cholesterol were determined. The results are given in Fig. 1.

The decrease in serum protein is only about ten per cent at *p*H 8.0, but increases with the more acid reaction of the serum reaching its maximum at *p*H 5.5. This *p*H is very close to the isoelectric point for the proteins, which may account for the great loss in protein. Probably the marked loss in serum lipids at *p*H 5.5 can also be ascribed to increased acidity of the serum only. In any case, a lipid protein complex with a relatively high lipid content is precipitated with bentonite at this pH. The most favourable conditions, *i.e.* the greatest possible removal of lipids with at the same time the smallest loss in proteins, thus seem to be obtained in a serum with a *p*H of 7.5 or more. The following experiments

therefore have been performed on sera with a slightly alkaline reaction. (*p*H about 8.0).

Effect of Different Bentonite Concentrations on Serum Protein, Lipid Phosphorus and Cholesterol. — The serum was diluted one to one with saline and equal parts of bentonite suspension in the concentrations 0.4, 0.6, 0.8, 1.0 and 1.2 per cent were added to the serum dilutions, thus making the final bentonite concentrations

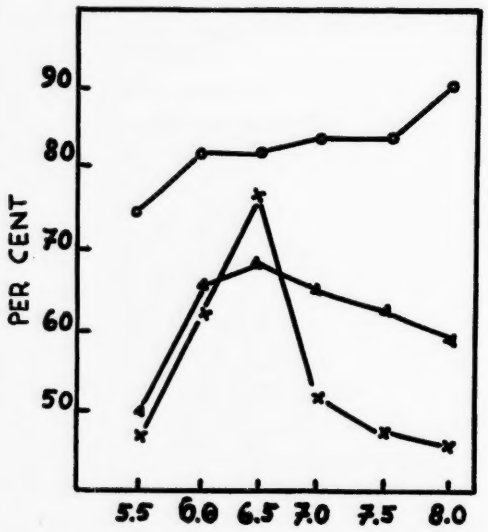


Fig. 1. — The effect of pH on bentonite treatment (concentr. 0,2 per cent). o——o protein, ×——× cholesterol, ▲——▲ lipid phosphorus.

0.2, 0.3, 0.4, 0.5 and 0.6 per cent. In this experiment the *p*H of the serum was 8.0. The serum-bentonite mixtures were treated as before and the serum protein, lipid phosphorus and cholesterol determined from the supernatant. The results are shown in Fig. 2 and represent the mean values of tests on five different sera.

The effect of the bentonite on the serum lipids is very marked. The cholesterol content of the serum decreases very rapidly and with the highest bentonite concentration (0.6 per cent) no remaining cholesterol could be demonstrated. Also the decrease in lipid phosphorus is pronounced, although it does not reach the same degree as that of the cholesterol. However, with a bentonite concentration of 0.6 per cent about 80 per cent of the lipid phosphorus

is removed. These results seem to support very well previous conceptions of the mode of association between lipids and proteins. Thus White (21) assumes that the cholesterol in the animal tissue occurs in a state of »weak combination» with fatty acids and lecithin, and later Tayeau (18) has presented evidence that cholesterol is mainly bound to the phosphatides (lipid-lipid linkage), and

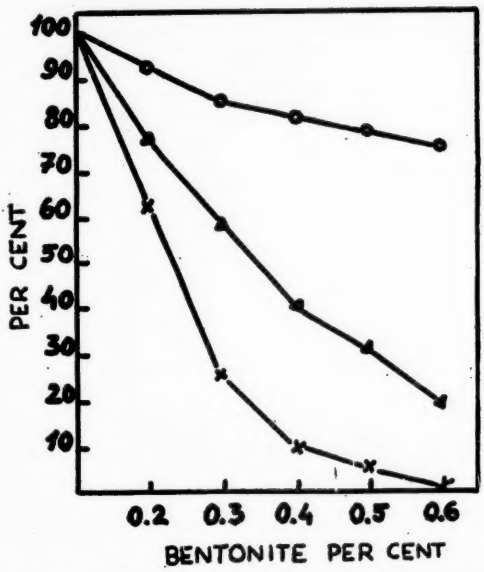


Fig. 2. — The effect of bentonite treatment on serum protein o——o, lipid phosphorus ▲——▲ and cholesterol ×——×.

phosphatides might form the link between cholesterol and proteins. The part of the phosphatides (about 20 per cent), with can not be removed by bentonite without an appreciable loss in proteins might be very closely bound to the proteins, probably as a part of the protein molecules. Although we have not analysed the composition of the remaining phospholipids in greater detail, the results seem to accord with those obtained by Blix with ether acetone extraction in the cold (1).

There is, apparently, also a loss in proteins, which, as was the case also with the lipids, is greater the more the concentration of the bentonite is increased. However, the loss in proteins does not

exceed 15 per cent, and is thus of an entirely different magnitude. In estimating the decrease in proteins it must be borne in mind that the values in fact show the total amount of »protein nitrogen», and thus part of the loss in protein (nitrogen) might be due to removal of nitrogen containing lipid components, such as choline and cholamine. The non-proteinN is not affected by the bentonite treatment.

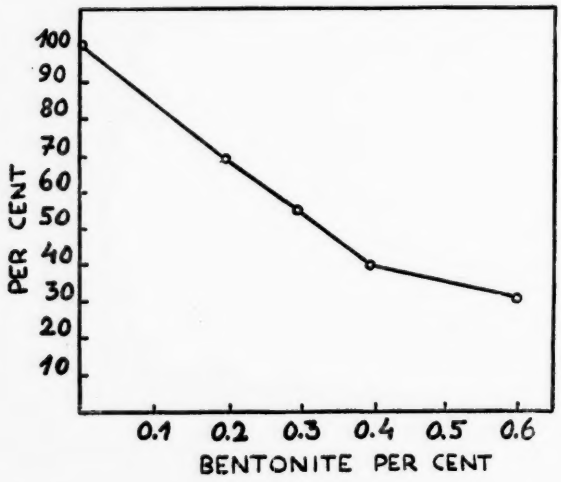


Fig. 3. — The effect of bentonite treatment on serum β-globulin concentration.

Effect of Bentonite on Electrophoresis of Serum. — Electrophoretic analyses were performed on untreated serum and on serum treated with bentonite in concentrations of 0.2, 0.3, 0.4 and 0.6 per cent.

The mobility of all the components always remained unchanged, thus indicating that no denaturation had occurred. The area under each peak was measured and calculated as a percentage of the corresponding areas of untreated serum. Thus it was observed that all the components except the β-globulin were practically unchanged. This component decreases with increased bentonite concentration. With a bentonite concentration of 0.6 per cent the β-globulin content of the serum was only 30 per cent of the original serum β-globulin. This is shown in Fig. 3.

These result are in accordance with those obtained by McFar-

lane (9) with lipid extraction with ether at —25° C. On the other hand, no loss in α-globulins could be demonstrated, which is the case if the lipids are extracted with acetone-ether in the cold according to Blix (1). The decrease in β-globulin is thus much more marked than is the loss in total protein. Since the lipids are supposed to occur mainly in the β-globulin fraction of the serum, this decrease may depend purely on the removal of lipoprotein.

Effect of Bentonite Treatment on the pH and the Ionic Strength of the Serum. — In several instances the *p*H and the ionic strength of the sera were determined before and after treatment with bentonite. No significant differences could, however, be demonstrated. It thus seems that the bentonite changes neither the *p*H of the serum nor the ionic strength.

SUMMARY AND CONCLUSION

According to the results presented above, most serum lipids can be removed at room temperature by absorption to bentonite sol from a dilute serum at *p*H 8.0, using a bentonite concentration of 0.6 per cent. With this treatment the serum cholesterol disappears entirely and the lipid phosphorus decreases to about 20 per cent. The decrease in serum protein as estimated by the Kjeldahl method is, on the other hand, only about 20 per cent, and electrophoretic analyses show that the only component practically affected is the β-globulin fraction of the serum.

In our opinion this method, thus, is very suitable for the removal of serum lipids for different purposes, and probably also for absorption of serologically non-specific serum fractions, the nature of which are still unknown.

The exact mechanism of the absorption has not been explained, but it seems possible that the effect of bentonite on the different serum components might be ascribed to the ion exchange-properties of the bentonite.

REFERENCES

1. Blix, G.: J. Biol. Chem. 1941:*137:*495.
2. Dawson, M. H., Olmstead, M., and Boots, R. H.: J. Immunol. 1932: *23:*187.
3. Dawson, M. H., Olmstead, M., and Boots, R. H.: ibid. 1932:*23:*205.

4. Dawson, M. H., Olmstead, M., and Jost, L.: ibid. 1934:*27:*355.
5. Hansen, A.: Acta Path. Microbiol. Scand. 1948:*25:*460.
6. Hardy, W. B., and Gardiner, S.: J. Physiol. 1910:*40:*LXVIII.
7. Kalbak, K.: Nord. Med. 1946:*31:*1997.
8. Kunkel, H. G., Ahrens, E. H., and Eisenmenger, W. S.: Gastroenterology 1948:*11:*499.
9. McFarlane, A. S.: Nature 1942:*149:*439.
10. Nicholls, E. E., and Stainsby, W. J.: J. Clin. Investig. 1931:*10:*323.
11. Oker-Blom, N.: Ann. Med. Exp. Biol. Fenn. 1948:*26:*77.
12. Oker-Blom, N., Nikkilä, E., and Kalaja, T.: ibid. 1950:28*:125.*
13. Oker-Blom, N.: ibid. (in press).
14. Oker-Blom, N.: ibid. (in press). — Acta Path. Microbiol. Scand. 1951 Suppl. *91:*123.
15. Packalen, Th.: Nord. Med. 1947:*33:*509.
16. Packalen, Th.: J. Bact. 1948:*56:*143.
17. Porsman, V. A.: Nord. Med. 1948:*40:*2147.
18. Tayeau, L.: Bull. Soc. Chim. Biol. 1941:*26:*287.
19. Wager, O.: Ann. Med. Exp. Biol. Fenn. 1950:*28:*Suppl. 8.
20. Wallis, A. D.: Am. J. Med. Sci. 1946:*212:*713, 716, 718. — 1947:*213:* 87, 94.
21. White, G.: Medic. Chron. 1908:47.
22. Winblad, S., and Edström, G.: Acta Path. Microbiol. Scand. 1948:*25:* 715.

FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

INDUCED RESISTANCE TO PENICILLIN

by

W. J. KAIPAINEN

(Received for publication September 14, 1951)

In several of Demerec's investigations (1, 2, 3, 4) into increased resistance, he emphasises the increased resistance of bacteria as resulting from spontaneous mutation and considers the role of the antibiotic a selective one. He has shown that two types of patterns exist, depending primarily on the antibiotic. The first is the penicillin type, where complete resistance is gradually attained through several genetic steps. The first step, due to the selective action of the antibiotic, yields material for the second step. This continues until the resistance is complete. In this first type no considerable increase in resistance, therefore, is attained by a single step. This type comprises penicillin, aureomycin, chloromycetin, terramycin and neomycin. The second is the streptomycin type, where resistance may be induced, apart from in the way described above, also by one single step.

Investigating *in vitro* the increased resistance of *Bacillus subtilis* to penicillin, the writer, however, made observations deviating from the above. The resistance of the *Bacillus subtilis* strains employed was increased by cultivating them in broth with a 2-fold serial dilution of penicillin. The method has been described in detail earlier (5, 6). After an incubation period of 24 hours the bacteria for the next series were taken from the first turbid tube. Increase in resistance after a single transfer was as follows (Table 1):

TABLE 1.

INCREASE IN THE RESISTANCE OF BACILLUS SUBTILIS STRAINS TO PENICILLIN AFTER A 24 HOUR-CULTIVATION IN BROTH CONTAINING PENICILLIN

Strain	Initial Sensitivity Unit/ml	After 24 Hours Unit/ml	Increase
Bacillus subtilis str. 1	0.03	1.9	64-fold
» 2	0.015	0.06	4- »
» 3	0.06	31.25	512- »
» 4	0.05	31.25	1024- »

The increase in resistance of Strain 2 is only 4-fold, as can be expected where increased resistance to penicillin is in question. Against this, the resistance of Strain 1 has increased considerably more. The increase in the resistance of Strains 3 and 4 is so considerable that it can be compared with an increase in the resistance of streptomycin type.

SUMMARY

Complete resistance of two *Bacillus subtilis* strains resulted after a single cultivation in broth containing penicillin, although the increase in bacterial resistance to penicillin usually takes place by multiple steps.

REFERENCES:

1. Bryson, V., and Demerec, M.: Ann. N. Y. Acad. Sci. 1950:*53:*283.
2. Demerec, M.: Proc. Nat. Acad. Sci., Wash. 1945:*31:*16.
3. Demerec, M.: J. Bact. 1948:*56:*63.
4. Demerec, M.: J. Clin. Invest. 1949:*28:*891.
5. Kaipainen, W. J.: Ann. Med. Exp. Biol. Fenn. 1950:*28:*222.
6. Kaipainen, W. J.: Ibid. 1951:*29:*Suppl. 1.

FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

EFFECT OF ANTIBIOTIC COMBINATIONS ON BACTERIAL RESISTANCE

by

W. J. KAIPAINEN

(Received for publication September 14, 1951)

A considerable disadvantage of antibiotics must be considered the fact that bacterial resistance to them may increase. The matter is further complicated in that increased resistance to one antibiotic may induce simultaneous increased resistance to other antibiotics as well (1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14). Aureomycin, chloromycin and terramycin seem to constitute a special group of their own in this respect (7, 9, 10, 11, 12). When the resistance of bacteria, especially of gram-negative rods (11), to one of the antibiotics in this group is increased, a simultaneous increase follows also in the resistance to the other two. But resistance to dihydrostreptomycin, however, usually remains unchanged or is slightly weakened. On the other hand, increased resistance primarily to dihydrostreptomycin results in unchanged or slightly reduced resistance to aureomycin, chloromycetin and terramycin. In an earlier paper (11) the author made a study as to whether this phenomenon possibly appears as the prevention of the increase in resistance with *E.coli* cultivated in broth containing small equal amounts, not inhibiting the growth of *E.coli*, of both dihydrostreptomycin and aureomycin (or, respectively, chloromycetin or terramycin). As the results of these experiments seemed positive, the author considered it worth while continuing the experiments reported in this paper, with the purpose of establishing whether experiments effected with more

extensive material confirm the author's observations regarding the prevention of increased bacterial resistance when combinations of antibiotics are employed.

PRESENT INVESTIGATION

The purpose was to establish the possible preventative effect of a combination of antibiotics of two different types, A (aureomycin, chloromycetin, terramycin) and B (dihydrostreptomycin), on the increase in bacterial resistance to these antibiotics when bacteria are cultivated from day to day in broth containing these antibiotics, alternatively, as follows:

I Continuously the same small amount, not inhibitory to bacterial growth, of A and B.

II Equal amounts of A and B in rising quantities.

III 1. A in rising quantities, with a continuous small amount of B.
2. B in rising quantities, with a continuous small amount of A.

Technique. — In sensitivity determinations a simple and quick serial dilution method was employed that the writer has employed previously (10, 11), the like of which has been employed for determinations of antibiotic concentrations by other authors as well (3, 6). Into each tube of the series was measured 1 ml of the bacterial suspension, prepared from 24-hours broth culture diluted to 10^{-4} with sterile broth (*p*H 7.4). 1 ml of the antibiotic solution (1 mg/ml) or suspension, respectively, was then added into the first tube and carefully mixed, after which 1 ml was transferred into the second tube, mixed carefully, and so on. Thus a 2-fold serial dilution of the antibiotic (500, 250, 125 μg/ml etc.) was obtained. Reading off the growth results after 24 hours, the antibiotic concentration of the last tube that had remained clear was=the sensitivity of the bacterium in question. The same method was applied to ascertain bacterial sensitivity to the combination of dihydrostreptomycin +aureomycin (or chloromycetin or terramycin), except that, instead of 1 ml of one antibiotic, 0.5 ml of each of the two antibiotics in question were measured into the first tube. In a sensitivity determination method of this kind, changes exceeding one tube can be considered as significant (11).

RESULTS

I

The first part of the investigation aims at comparing how high the bacterial resistance will rise when bacteria are transferred from day to day *1.* into broth containing a constant amount, not sufficient to prevent bacterial growth, of one antibiotic (dihydrostreptomycin, aureomycin, chloromycetin or terramycin), *2.* into broth containing simultaneously equally large amounts as in Item *1.* of two antibiotics of different types, dihydrostreptomycin and aureomycin (or chloromycetin or terramycin).

The antibiotic concentration in broth was 1 μg/ml, except in the cases where this concentration was so high that it inhibited the growth of the bacteria when a concentration was employed that was half the combination concentration inhibiting the growth. After each 24 hours' growth a loopful of the bacterial mixture was transferred to a fresh lot of broth and antibiotic. This was repeated ten times, after which sensitivity determinations were effected. As a control, the same bacterium was cultivated simultaneously by daily transfer in broth only.

The small antibiotic concentration in broth was selected to correspond to the possible low antibiotic concentrations present in unfavourable conditions *in vivo*, where micro-organisms have a chance of becoming resistant.

The writer's interest was focussed on the gram-negative rods, as it was with them that the difference in behaviour of dihydrostreptomycin from the aureomycin, chloromycetin and terramycin group had been established (11). There seem to be strains among the gram-negative rods that are close to the resistance limit with regard to aureomycin, chloromycetin and terramycin, for which reason, in unfavourable conditions, there is a possibility of resistant strains developing *in vivo*. Although *Proteus vulgaris* does not seem to be sensitive to the antibiotics in current use, two *Proteus vulgaris* strains have nevertheless been included.

Combination Aureomycin + Dihydrostreptomycin (Table 1). — With *E.coli*, *A.aerog.* and *S. typhi* strains the combination concentration inhibiting bacterial growth was so low that corresponding concentrations in blood are possible when antibiotics are used in normal dosage. Growth-inhibiting concentration of the combina-

tion seems to be determined by the most effective component, whether aureomycin or dihydrostreptomycin. In some cases a slight aggregation of effect is observable, as with the first four *E.coli* strains. Similarly, the *Proteus vulgaris* 1492, resistant to aureomycin and dihydrostreptomycin, seems to be less resistant to their combination.

After 10-day cultivation bacterial resistance to the combination as a rule increases, but the increase seems slight. Increase in resistance with *E.coli* and *S.typhi* strains is so slight that these strains can still be considered sensitive *in vitro* to the combination aureomycin + dihydrostreptomycin. In the combination the resistance to each of the two components, aureomycin and dihydrostreptomycin, increases somewhat but seems generally to remain at a lower level than the increase in resistance in Columns I and II, where the bacteria were cultivated in broth containing, in the former case aureomycin, and in the latter dihydrostreptomycin. The increase in resistance to dihydrostreptomycin (Column III) seemed less marked than to aureomycin.

Hence, it seems that bacteria can be cultivated in broth without any considerable increase in resistance, for several days, at such concentrations of the combination aureomycin + dihydrostreptomycin as do not suffice to inhibit bacterial growth.

Combination Chloromycetin + Dihydrostreptomycin (Table 2). — Bacterial resistance to the combination chloromycetin + dihydrostreptomycin also seems to be determined by the more effective component, which with regard to these strains is chloromycetin in the majority of cases.

After 10-day cultivation the resistance of practically every bacterial strain to the combination chloromycetin + dihydrostreptomycin increased, and the increase seems to be slightly greater than in the corresponding experiment with aureomycin and dihydrostreptomycin (Table 1). The increase in resistance to the components chloromycetin and dihydrostreptomycin (Table 2, Column III), however, seems less marked than the increase in resistance in Columns I and II, where the bacteria were cultivated in broth containing, in the former case chloromycetin, and in the latter dihydrostreptomycin.

Combination Terramycin + Dihydrostreptomycin (Table 3). — Bacterial resistance to the combination terramycin + dihydro-

TABLE 1

INCREASE IN BACTERIAL RESISTANCE AFTER 10 DAYS CULTIVATION IN BROTH CONTAINING ANTIBIOTIC AS FOLLOWS:

I AUREOMYCIN
II DIHYDROSTREPTOMYCIN
III AUREOMYCIN + DIHYDROSTREPTOMYCIN (THE SAME AMOUNTS AS IN I AND II)

Organism	Amount of drug in Broth μ g/ml	I Sensitivity to Aureomycin μ g/ml			II Sensitivity to Dihydrostreptomycin μ g/ml			III Sensit. to Comb. Aureom. + Dihydrostrept.			Sensit. to Aureom. after		Sensit. to Dihydrostr. after	
		before	after	Increase	before	after	Increase	before	after	Increase	Expos.	Increase	Expos.	Increase
E.coli 11	0.5	7.8	125	16-f.	7.8	>500	>64-f.	1.9	1.9	1-f.	31.2	4-f.	31.2	4-f.
E.coli 24	0.5	7.8	62.5	8	1.9	62.5	32	1.9	1.9	1	3.9	0.5	15.6	8
E.coli 19,936	0.5	3.9	62.5	16	15.6	62.5	4	1.9	1.9	1	3.9	1	31.2	2
E.coli 19,983	0.5	1.9	15.6	8	7.8	125	16	0.97	3.9	4	7.8	4	15.6	2
E.coli 22,979	0.5	3.9	62.5	16	3.9	125	32	3.9	7.8	2	15.6	4	7.8	2
A.aerog. 899	1	3.9	31.2	8	7.8	250	32	3.9	15.6	4	62.5	16	15.6	2
A.aerog. 1,120	1	7.8	250	32	7.8	31.2	4	3.9	15.6	4	62.5	8	7.8	1
S.typhi ö	1	3.9	15.6	4	31.2	500	16	3.9	7.8	2	3.9	1	31.2	1
S.typhi 38	0.5	0.97	15.6	16	125	500	4	0.97	7.8	8	7.8	8	500	4
Prot.vulg. 1,423	1	>500	>500	—	31.2	250	8	31.2	125	4	>500	—	250	8
Prot.vulg. 1,492	1	500	500	1	62.5	250	4	15.6	62.5	4	500	1	250	4

TABLE 2

INCREASE IN BACTERIAL RESISTANCE AFTER 10 DAYS CULTIVATION IN BROTH CONTAINING ANTIBIOTIC AS FOLLOWS:

I CHLOROMYCETIN
II DIHYDROSTREPTOMYCIN
III CHLOROMYCETIN + DIHYDROSTREPTOMYCIN (THE SAME AMOUNTS AS IN I AND II)

Organism	Amount of drug in Broth μ g/ml	I Sensitivity to Chloromycetin μ g/ml			II Sensitivity to Dihydrostreptomycin μ g/ml			III Sensit. to Comb. Chlorom. + Dihydrostrept.			Sensit. to Chloromycet. aft.		Sensit. to Dihydrostr. aft.	
		before	after	Increase	before	after	Increase	before	after	Increase	Expos.	Increase	Expos.	Increase
E.coli 11	0.5	3.9	62.5	16-f.	7.8	>500	>64-f.	3.9	3.9	1-f.	7.8	2-f.	15.6	2-f.
E.coli 24	0.5	3.9	62.5	16	1.9	62.5	32	3.9	3.9	1	7.8	2	31.2	16
E.coli 19,936	0.5	3.9	31.2	8	15.6	62.5	4	3.9	15.6	4	7.8	2	15.6	1
E.coli 19,983	0.5	1.9	7.8	4	7.8	125	16	1.9	7.8	4	3.9	2	31.2	4
E.coli 22,979	0.5	1.9	31.2	16	3.9	125	32	3.9	7.8	2	15.6	8	15.6	4
A.aerog. 899	1	7.8	125	16	7.8	250	32	3.9	31.2	8	125	16	31.2	4
A.aerog. 1,120	1	3.9	250	64	7.8	31.2	4	3.9	31.2	8	125	32	31.2	4
S.typhi ö	1	1.9	1.9	1	31.2	500	16	1.9	3.9	2	3.9	2	31.2	1
S.typhi 38	0.5	0.97	1.9	2	125	500	4	0.97	1.9	2	1.9	2	62.5	0.5
Prot.vulg. 1,423	1	15.6	250	16	31.2	250	8	15.6	125	8	125	8	62.5	2
Prot.vulg. 1,492	1	31.2	250	8	62.5	250	4	15.6	125	8	125	4	125	2

TABLE 3

INCREASE IN BACTERIAL RESISTANCE AFTER 10 DAYS CULTIVATION IN BROTH CONTAINING ANTIBIOTIC AS FOLLOWS:

I TERRAMYCIN
II DIHYDROSTREPTOMYCIN
III TERRAMYCIN + DIHYDROSTREPTOMYCIN (THE SAME AMOUNTS AS IN I AND II)

Organism		Amount of drug in Broth μ g/ml	I			II			III						
			Sensitivity to Terramycin μ g/ml			Sensitivity to Dihydrostreptomycin μ g/ml			Sensit. to Comb. Terram. + Dihydrostrept.			Sensit. to Terramycin after		Sensit. to Dihydrostr. after	
			before	after	Increase	before	after	Increase	before	after	Incr.	Expos.	Increase	Expos.	Increase
E.coli	11	0.5	3.9	7.8	2-f.	7.8	>500	>64-f.	0.97	0.97	1-f.	3.9	1-f.	31.2	4-f.
E.coli	24	0.5	0.97	15.6	16	1.9	62.5	32	0.97	3.9	4	7.8	8	62.5	32
E.coli	19,936	0.5	1.9	7.8	4	15.6	62.5	4	0.97	7.8	8	3.9	2	62.5	4
E.coli	19,983	0.5	0.97	7.8	8	7.8	125	16	0.97	3.9	4	7.8	8	31.2	4
E.coli	22,979	0.5	0.97	15.6	16	3.9	125	32	0.97	1.9	2	1.9	2	15.6	4
A.aerog.	899	1	3.9	31.2	8	7.8	250	32	3.9	7.8	2	15.6	4	15.6	2
A.aerog.	1,120	1	3.9	31.2	8	7.8	31.2	4	3.9	15.6	4	31.2	8	7.8	1
S.typhi	ö	1	3.9	31.2	8	31.2	500	16	3.9	7.8	2	15.6	4	125	4
S.typhi	38	0.5	3.9	15.6	4	125	500	4	1.9	7.8	4	7.8	2	500	4
Prot.vulg.	1,423	1	250	500	2	31.2	250	8	31.2	125	4	500	2	250	8
Prot.vulg.	1,492	1	250	500	2	62.5	250	4	31.2	125	4	250	1	62.5	1

streptomycin seems to take the same course as to the previous combinations (Tables 1 and 2). The more effective component, terramycin, determines the inhibitory level with strains other than *Proteus vulgaris*, which are more sensitive to dihydrostreptomycin. In some cases a slight aggregation of effect occurs.

After 10-day cultivation bacterial resistance to the combination terramycin + dihydrostreptomycin increases in the same proportions as in the previous combinations (Tables 1 and 2).

Taken as individual results, the majority of the final values in Columns I and III are within the range of the potential error of the method, but taken together and interpreted as a deviation in the same direction they seem to indicate that increased resistance to terramycin in the combination terramycin + dihydrostreptomycin (Column III) is less than the increase in resistance to terramycin when the bacteria were cultivated in broth containing terramycin alone. The effect of the combination terramycin + dihydrostreptomycin on the increase of resistance to dihydrostreptomycin, on the other hand, is fairly marked with many strains.

II

In this part of the investigation two *E.coli* strains were cultivated in broth with rising concentrations of antibiotic combinations. Using the serial 2-fold dilution method reported above, the bacteria were taken in daily transfers for the next series from the first turbid tube. This being so, the most resistant individuals only persisted in the daily cultures. After 13 transfers sensitivity determination to the different components of the combination was also effected. Simultaneously the same strains were cultivated in broth containing only one antibiotic in rising concentrations.

TABLE 4

EFFECT OF COMBINATION AUREOMYCIN + DIHYDROSTREPTOMYCIN ON THE INCREASE IN RESISTANCE OF E.COLI STRAINS 11 AND 24

Organism and Strain	Cultivated in Broth with *Rising* Concentrations of	Sensitivity μ g/ml		
		Aureomycin	Dihydro-streptomycin	Aureomycin + Dihydrostrept.
E.coli 11	0	7.8		
	aureomycin	125		
»	0		7.8	
	dihydrostrept.		>500	
»	0			0.97
	aureomycin + dihydrostrept.	7.8	7.8	7.8
E.coli 24	0	7.8		
	aureomycin	62.5		
»	0		3.9	
	dihydrostrept.		>500	
»	0			0.97
	aureomycin + dihydrostrept.	7.8	7.8	7.8

Aureomycin + Dihydrostreptomycin (Table 4). — When *E.coli* was cultivated in broth containing aureomycin only the resistance of Strain 11 increased from 7.8 μg/ml → 125 μg/ml. Simultaneously, in the combination series aureomycin + dihydrostreptomycin, sensitivity remained unchanged. The results with *E.coli* Strain 24 were similar. The preventative effect of the combination on the

increase in resistance to dihydrostreptomycin was distinct with both *E.coli* strains, compared with the increase in resistance that followed cultivation in broth containing dihydrostreptomycin. The extended cultivation period and rising concentrations resulted in the resistance to dihydrostreptomycin of *E.coli* Strain 24 increasing from 3.9 μg/ml > 500 μg/ml, due to which the effect of the combination preventing the increase in resistance seems more marked than in the corresponding experiments in Table 1.

TABLE 5

EFFECT OF COMBINATION CHLOROMYCETIN + DIHYDROSTREPTOMYCIN ON THE INCREASE IN RESISTANCE OF E.COLI STRAINS 11 AND 24

Organism and Strain	Cultivated in Broth with *Rising* Concentrations of	Sensitivity μ g/ml		
		Chloromycetin	Dihydrostreptomycin	Chloromycetin + Dihydrostrept.
E.coli 11	0	3.9		
	chloromycetin	62.5		
»	0		7.8	
	dihydrostrept.		>500	
»	0			1.9
	chloromycetin + dihydrostrept.	7.8	7.8	7.8
E.coli 24	0	3.9		
	chloromycetin	125		
»	0		3.9	
	dihydrostrept.		>500	
»	0			1.9
	chloromycetin + dihydrostrept.	7.8	15.6	7.8

Chloromycetin + Dihydrostreptomycin (Table 5). — The results in these experiments were on the same lines as with the combination aureomycin + dihydrostreptomycin. The preventative effect on the increase in resistance of the combination chloromycetin + dihydrostreptomycin is somewhat more distinct in Table 5 than that of the corresponding combination in Table 2.

Terramycin + Dihydrostreptomycin (Table 6). — The preventative effect on the increase in resistance of the combination terramycin + dihydrostreptomycin was very marked with dihydro-

TABLE 6

EFFECT OF COMBINATION TERRAMYCIN + DIHYDROSTREPTOMYCIN ON THE INCREASE IN RESISTANCE OF E.COLI STRAINS 11 AND 24

Organism and Strain	Cultivated in Broth with *Rising* Concentrations of	Sensitivity μ g/ml		
		Terramycin	Dihydrostreptomycin	Terramycin + dihydrostrept.
E.coli 11	0	3.9		
	terramycin	7.8		
»	0		7.8	
	dihydrostrept.		>500	
»	0			0.97
	terramycin + dihydrostrept.	7.8	7.8	1.9
E.coli 24	0	0.97		
	terramycin	62.5		
»	0		3.9	
	dihydrostrept.		>500	
»	0			0.97
	terramycin + dihydrostrept.	7.8	7.8	7.8

streptomycin. The same applies to terramycin with *E.coli* Strain 24. The increase resistance to terramycin of *E.coli* Strain 11 was the same both with the drug alone and in combination. The increase was so small in both cases that the change falls within the range of the potential error.

III

In the combination experiments reported above the dihydrostreptomycin concentration of the broth was always equal to the aureomycin, chloromycetin or terramycin concentration. This part of the investigation was carried out in order to establish whether the increase in resistance to aureomycin is prevented by dihydrostreptomycin when the broth contains a constant amount of it, less than the original amount of aureomycin which is administered in rising quantities.

A 2-fold serial dilution of aureomycin was made into broth of a dihydrostreptomycin concentration of 0.5 μg/ml, and *E.coli* strain 11 was cultivated in this, taking daily the bacteria for the

next series always from the first turbid tube of the dilution series. Increase in aureomycin resistance was compared with the increase in resistance in similar series without dihydrostreptomycin. It appeared that aureomycin resistance increased step-wise in both series in the daily transfers, approximately in the same degree.

Similar experiments were made to ascertain 1) the effect of a small amount of dihydrostreptomycin on the increase in resistance to chloromycetin and terramycin and 2) the effect of small amounts of aureomycin, chloromycetin and terramycin on the increase in resistance to dihydrostreptomycin. However, no inhibition of the increase in resistance was verified.

It seemed, therefore, that the mutual concentration of the components in the combination was of importance in achieving a positive result.

SUMMARY AND CONCLUSIONS

As the resistance of gram-negative rods 1) with resistance to aureomycin, chloromycetin or terramycin increasing, may decrease to dihydrostreptomycin, or 2) with resistance increasing primarily to dihydrostreptomycin, may slightly decrease to aureomycin, chloromycetin or terramycin, experiments were carried out to establish whether *in vitro* the combination dihydrostreptomycin + aureomycin, chloromycetin or terramycin prevents the increase in the resistance of some *E.coli*, *A.aerogenes*, *S.typhi* and *Proteus vulgaris* strains to these antibiotics.

At least a partial inhibition of the increase in resistance seemed distinct, with *E. coli* strains in particular. The results were roughly the same with all combinations. The experiment carried out with one *E.coli* strain showed that, the mutual concentration of the components in the combination was of importance in achieving a positive result.

The results seem to indicate that at least a theoretical basis exists for the use of the combinations

aureomycin + dihydrostreptomycin
chloromycetin + »
terramycin + »

in therapy, whenever an increase in the resistance of gramnegative rods either to aureomycin, chloromycetin, terramycin or dihydrostreptomycin is anticipated.

REFERENCES

1. Dowling, H. F., Hirsh, H. L., and O'Neil, C. B.: J. Clin. Invest. 1946:*25:*665.
2. Eisman, P. C., Marsh, W. S., and Mayer, R. L.: Science 1946:*103:*673.
3. Frisk, A. R., and Tunevall, G.: Scand. J. Clin. Lab. Invest. 1950:*2:*26.
4. Gocke, T. M., Jackson, G. G., Wilcox, C., and Finland, M.: Ann. N. Y. Acad. Sci. 1950:*53:*297.
5. Graessle, O. E., and Frost, B. M.: Proc. Soc. Exper. Biol., N.Y. 1946: *63:*171.
6. Herrell, W. E., and Heilman, F. R.: Proc. Mayo Clin. 1949:*24:*157.
7. Herrell, W. E., Heilman, F. R., and Wellman, W. E.: Ann. N.Y. Acad. Sci. 1950:*53:*448.
8. Kelner, A., and Morton, H. E.: J. Bact. 1947:*53:*695.
9. Kaipainen, W. J.: Ann. Med. Exper. Biol. Fenn. 1951:*29:*100.
10. Kaipainen, W. J.: Ibid. 1951:*29:*247.
11. Kaipainen, W. J.: Ibid. 1951:*29:*Suppl. 1.
12. Pansy, F. E., Khan, P., Pagano, J. F., and Donovick, R.: Proc. Soc. Exp. Biol., N. Y. 1950:*75:*618.
13. Rake, G., McKee, C. M., Pansy, F. E., and Donovick, R.: Ibid. 1947:*65:*107.
14. Sullivan, M., Stahly, G. L., Birkeland, J. M., and Myers, Wm. G.: Science 1946:*104:*397.

FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

RHEUMATIC SERA AND CORTISONE TREATMENT

by

E. J. JOKINEN

(Received for publication September 29, 1951)

It is well known that serum changes occur in patients suffering from rheumatoid arthritis. The following is a report on a method by which an obvious difference between the sera of patients suffering from this disease and those of healthy subjects was obtained.

METHOD

2.5 ml of 94 per cent ethyl alcohol was measured into a test tube. 0.25 ml of the serum to be examined was pipetted into the same tube for precipitation. The test tube was placed in a refrigerator for 24 hours. After this it was centrifuged for 5 minutes at 2500 RPM. The alcohol was poured off and the sediment allowed to dry at room temperature for 15 minutes with the tube turned upside down. 1 ml of concentrated sulphuric acid was introduced into the tube and the tube was kept at 56° C in a water bath for 5 minutes. The sediment was gradually dissolved, and the sulphuric acid was turned brown at the same time.

RESULTS

By this method, a series of 15 »normal» and 15 rheumatic sera was tested. The former had been obtained from blood donors without any previous selection and the latter from hospital patients whose only clinical diagnosis was rheumatoid arthritis.[1] Both groups of

[1] I wish to express my gratitude to Prof. P. Soisalo, M.D., for placing the clinical material from the Kivelä Hospital, Helsinki at my disposal.

sera were treated parallelly at the different stages, so as to make the method as identical as possible for both.

It was found that in the tubes containing the sediment of a rheumatic serum the darkening of the sulphuric acid was distinctly more marked and the sediment dissolved faster than in the tubes containing the sediment of a normal serum. The faster the sediment dissolved the darker the sulphuric acid became. When the two lots were placed separately in order of darkness, it was found that the difference between the »normal» and the rheumatic lot was not

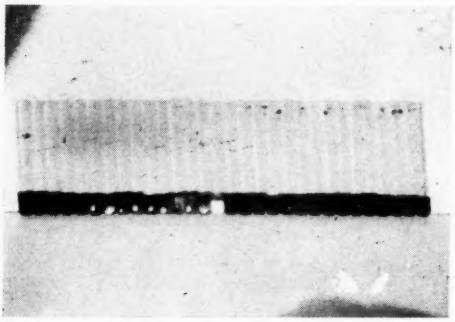


Fig. 1. — The reactions in order of darkness; to the left, those given by the »normal»; to the right, those given by the rheumatic sera.

absolute: the very darkest of the former were darker than the lightest among the latter. Taking the series as a whole, however, the difference was quite apparent (Fig. 1).

The above test series was effected three times with the same sera. Their order of darkness varied at different times. However, it was found that the same sera generally gave the lightest reaction on every occasion, and the same sera the darkest. This could be seen both in the »normal» and in the rheumatic lot (Table). Evidently due to technical differences, the darkness of the series seen as a whole was not exactly the same at different times.

In a comparison of the intensity of colour and the clinical severity of disease, attention was attracted by the fact that the two rheumatic sera that had given the lightest colour (R14 and R15 in the table) came from patients in whom the disease seemed exceedingly mild in form, e.g., the sedimentation rate for both of them was 17 mm/h. Three sera that generally remained at the

TABLE

1st Series	2nd Series	3rd Series
R1	R6	R1
R2	R2	R10
R3	R3	R2
R4	R1	R6
R5	R7	R5
R6	R12	N2
R7	R10	R9
N1	R5	R12
R8	R4	R4
R9	R11	R8
R10	R8	N1
N2	R9	R11
R11	N1	R7
R12	R13	R3
N3	N3	N3
R13	R14	R13
R14	R15	R14
N4	N2	N11
N5	N7	N7
N6	N8	N9
N7	N11	N6
R15	N5	N5
N8	N10	N8
N9	N9	N12
N10	N12	N13
N11	N13	N10
N12	N4	N4
N13	N15	R15
N14	N14	N14
N15	N6	N15

Sera in the order of darkness of the reactions given by them. R1—R15 rheumatic sera. N1—N15 »normal» sera.

darker end (R1—R3 in the table), again, came from patients whose sedimentation rate varied within the limits 97—137 mm/h, and with whom the disease seemed very active otherwise also. According to their own information, two (N2 and N3 in the table) of the three blood donors whose sera gave the most intense colour of those in the normal lot had previously been treated for rheumatoid arthritis, although they were now healthy.

To ascertain whether the difference observed between normal and rheumatic sera persists even after cortisone treatment, when the patient's condition has improved clinically, the sera of three patients were compared, using the method described above, before cortisone treatment and after a treatment of 9—11 days. Used as controls were the sera of two patients receiving other treatment, It was found that cortisone had changed the reaction of the sera distinctly in a normal direction, which was evident from the fact

Fig. 2. — Reactions of rheumatic sera prior to cortisone treatment Nos. 3, 4, 5 to the right and after it Nos. 3, 4, 5 in the middle. Reactions of control sera prior to treatment Nos. 1, 2 in the middle and after it Nos. 1, 2 to the left.

that the sulphuric acid remained considerably lighter (Fig. 2). On the other hand, there was no change in the mode of reaction of the sera of patients who had received other treatment. The condition of all cortisone patients improved both subjectively and objectively. With one of them, the sedimentation rate dropped between two tests from 60 to 25 mm/h, with two of them there was no striking change in sedimentation rate.

SUMMARY

Sediments precipitated by 94 per cent ethyl alcohol from sera of patients suffering from rheumatoid arthritis generally dissolve more rapidly in concentrated sulphuric acid and in the process of dissolving give a darker brown colour than sediments precipitated from normal sera.

The above difference seems to disappear with cortisone treatment.

D-LICHESTERIC ACID

EFFECT *IN VIVO* ON PIGMENTED MICE WITH INOCULATION TUBERCULOSIS

by

K. O. VARTIA and LEO TERVILÄ

(Received for publication October 16, 1951)

The results obtained regarding the effect of protolichesteric and lichesteric acids on the growth of tubercle bacillus *in vitro* vary a great deal. With glycerin broth as the nutrient medium, inhibitory titres of 1:600,000—1:1,000,000 (1), 1:25,000—1:64,000 (7) and 1:40,000 (6) have been obtained, while with Dubos's medium the values have been considerably lower, 1:4,000—1:10,000 (7) and 1:5,000 (8), and with Kirchner's modification 1:5,000 (8). The factor causing the loss in efficiency is evidently the serum albumin which the latter two nutrient media contain and which is known to inactivate the physiological effects of compounds of aliphatic (fatty) acid type in general (4). As regards d-lichesteric acid, a serum addition of 10 per cent is capable, at the titre of 1:5,000, of destroying both its haemolytic effect and the antibiotic effect it has on Gram-positive cocci (9).

In the work here presented the intention was to study the possible effect of d-lichesteric acid on inoculation tuberculosis *in vivo*, also in combination with streptomycin. In spite of the obvious inactivating influence of human serum such an experiment has been considered justifiable, as, e.g., usnic acid, used in combination with streptomycin, has been found to affect the course of tuberculosis in guinea-pigs (5), in spite of the fact that its inhibitory titres against TB, on changing from a synthetic culture medium to a serum-containing medium, drop to less than 10 per cent of the original (7).

Pigmented mice susceptible to tuberculosis (strain C 57) were

used as the test animals; they were infected by injecting intravenously 0.25 cc of homogenous bacterial suspension grown for 8 days in Dubos's culture medium. The TB strain employed was a fresh strain pathogenic to guinea-pigs, isolated from a routine sample. The test animals were divided up into four groups: getting 1) from the day following infection and onwards, 0.2 cc of Na-salt solution (2 mg) of 1 per cent lichesteric acid administered daily straight into the stomach through a cannula inserted under aether anaesthesia of a couple of minutes (8 mice) *; 2) for a corresponding period instead of lichesteric acid streptomycin was administered subcutaneously, 1 mg per day (8 mice); 3 for a corresponding period, both drugs simultaneously, lichesteric acid 2 mg and streptomycin 1 mg daily (7 mice); 4) control animals, no drugs (5 mice).

The mice were weighed every five days, and an autopsy was performed after 5 weeks. The average variations in weights and the occurrence of tuberculous pulmonary changes in the various groups of treatment are given in Tables 1 and 2. According to the

TABLE 1

AVERAGE VARIATION IN WEIGHT AND THE OCCURENCE OF TUBERCULOUS PULMONARY CHANGES IN GROUPS OF MICE INOCULATED WITH A FRESH TB STRAIN AND TREATED DIFFERENTLY

Days	5	10	15	20	25	30	35	Change in Weight	Tuberculous Changes Large	Slight	None
Lichesteric acid 2 mg/day	22.0	22.0	21.3	20.8	21.0	21.0	20.8	— 1.2	••	••	
	13.8	14.0	16.0	15.0	15.5	17.0	16.2	+ 2.4	••	••	
Lichesteric acid 2 mg/day + streptomycin 1 mg/day	20.7	22.0	20.3	20.7	22.3	21.3	21.3	+ 0.6		•••	
	21.0	21.7	21.0	20.3	20.6	22.0	21.3	+ 0.3		••	oo
Streptomycin 1 mg/day	22.4	21.0	22.0	19.0	20.4	21.5	19.0	— 3.4		••	o
	13.4	13.3	15.0	14.0	15.0	16.8	15.0	+ 1.6		••	ooo
Control animals ..	20.0	22.0	20.4	22.0	22.0	21.4	20.0	± 0	••	•••	

* Attempts were also made to inject lichesteric acid, in the form of Na-salt, subcutaneously and intramuscularly in 1 per cent, 0.5 per cent and 0.25 per cent solutions, but it caused necroses slow to heal.

TABLE 2

AVERAGE VARIATIONS IN WEIGHT IN GROUPS OF MICE INOCULATED WITH A FRESH TB STRAIN AND TREATED WITH LICHESTERIC ACID AND STREPTOMYCIN

Days	5	10	15	20	25	30	35	Change in Weight
Lichesteric acid 2 mg/day	17.9	18	18.6	17.9	18.2	19.0	18.5	+ 0.6
Streptomycin 1 mg/day	17.9	17.1	18.5	19.6	17.7	19.1	17.0	— 0.9

latter, the average weight of mice receiving streptomycin dropped during the treatment, while that of mice receiving lichesteric acid rose, in spite of larger tuberculous pulmonary changes. The division of the pulmonary changes into three groups was based on microscopic examination of tissue preparations (the number and extent of cheesy necroses). The technique and evaluation of the results is in keeping, in the first place, with those applied by Hurni and his co-workers (3).

DISCUSSION

d-Lichesteric acid was not found to affect the course of inoculation tuberculosis in mice, whereas in the groups treated with streptomycin alone and with both lichesteric acid and streptomycin the course of tuberculosis was distinctly retarded. A study of the average weights of the different groups suggests that the mice that had received lichesteric acid reacted most positively, which is perhaps connected with the established resorption-promoting effect of lichesteric acid (2).

REFERENCES

1. Cavallito, C. J., Fruehauf, D. Mck., and Bailey, J. H.: J. Am. Chem. Soc. 1948:*70*:3724.
2. Fischer, R., and Toth, D.: Arch. Exp. Path. 1938:*190*:500.
3. Hurni, H., Hirt, R., and Ragaz, L.: Schw.Z. Path. Bakt. 1951 XIV:17.
4. MacLeod, Colin M.: Evaluation of Chemotherapeutic Agents, New York 1949, page 51.
5. Marshak, A., and Kuschner, M.: Publ. Health Rep. 1950:*66*:131.
6. Shibata, S., and Miura, Y.: Jap. Med. J. 1948:*1*:518.
7. Stoll, A., Brack, A., and Renz, J.: Schw.Z. Path. Bakt. 1950:XIII:752.
8. Vartia, K. O.: Ann. Med. Exp. Biol. Fenn. 1950:Suppl. No. 7:67.
9. Vartia, K. O.: unpublished.

FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

OCCURRENCE OF AN EXCEEDINGLY WEAK »A» BLOOD GROUP PROPERTY IN A FAMILY

by

EERO ESTOLA and JAAKKO ELO

(Received for publication October 17, 1951).

Fischer and Hahn (3) were the first to describe, in 1951, an exceedingly weak blood group A, in which the agglutinability, absorptive capacity and avidity of the cells were inferior to those of A_2 cells.

Friedenreich (4) found in 1936, among 4000 blood samples studied, six that reacted more weakly than the A_2 group, and called this the A_3 blood group. He made a genetic study of these A_3 families and, among the 260 persons examined, 46 belonged to the group A_3-, and 3 to A_3B-group. The A_3-quality was hereditary, like the 3rd allele A gene, being recessive for the A_1 and A_2 groups. Friedenreich characterized the properties of blood group A_3 as follows:

> Typical of the cells is an agglutination developing slowly on the slide, with some not particularly small but fragile agglutinates among the generally unagglutinated cells. Agglutination titre is lower or at the most as high as that of A_2B cells. The absorptive capacity of A_3 is weaker than that of A_2, but stronger than that of A_2B. The α_1 agglutinin, active at room temperature, is never present in the serum, and the cold agglutinins active at 2—5°C occur seldom.

In 1940 Hirszfeld and Amzel (8) published their investigation on a weak A blood group, which they termed A_4. On the basis of this and previously published investigations into the weak A groups,

they proposed that these groups be divided up into A_3, A_4 and A_5. At the same time they discussed the position of these groups as transition forms between 0 and A_2.

Gammelgaard and Marcussen (6), in 1940, found a very weakly reacting A blood group which they too termed A_4. In his more detailed investigation in 1942 Gammelgaard (5), however, changed this blood group to A_5, describing in addition, on the basis of his own material, the largest published hitherto, the most typical modes of reaction of A blood group properties weaker than A_2:

A_3: reactions similar to those described by Friedenreich. The cells agglutinate with anti-O serum more strongly than the A_2 cells. A antigen is present in the saliva of those of secretor type, less in amount than with A_2 subjects. The frequency of A_3 was $1^0/_{00}$ of all those belonging to the A Group.

A_4: Agglutinability and absorption capacity slightly inferior to A_2B. The agglutination picture typical of A_3 is missing. Cells agglutinate with anti-O serum as intensely as the A_3 cells. In four cases, out of the 10 samples studied, an irregular α_1 agglutinin active at room temperature was present in the serum. The A antigen content of the saliva was very small. The material comprised 3 families, with a total of 21 A_4 persons.

A_5: Agglutinability and absorption capacity still weaker than with A_4. Agglutination with anti-O serum as marked as with A_2 cells. In 13 cases out of the 15 samples studied, α_1 agglutinin active at room temperature was present in the serum, and in 2 cases α_2 agglutinin. Very difficult to prove A antigen in the saliva, and the division into secretors and non-secretors impossible. The material consisted of 3 families, with a total of 27 A_5 persons.

A_X: Agglutinability similar to that of A_5B. Incapable of absorbing anti-A serum. Does not agglutinate in anti-O serum. No anti-A agglutinins in the serum. A-antigen content of saliva equal to that of A_1 and A_2 secretors. The material comprised one A_X subject; his close relations did not possess the property.

The weak A properties examined, with a single exception, were inherited according to Mendel's law, being recessive with regard to A_1 and A_2.

Moureau (14, 15), in his investigations based on probability calculus, came to the conclusion that the differences between A_1, A_2 and A_3 are qualitative.

In 1948 Jonsson and Fast (9) published their investigation into the weak A blood group, which they termed A_6. The cells of this group did not agglutinate at all in anti-A sera, whereas they showed a tendency to rouleau formation. The absorption capacity

of the cells was approximately the same as or slightly inferior to that of A_2B cells. α_1 cold agglutinin was irregularly present in the serum. The material comprised 3 persons belonging to one and the same family.

Further cases of the occurrence of weak A blood groups have been published by Wiener & Silverman (17), Morawiecki (12), Hartmann, Hadland & Lundevall (7), Kammann (10), Dahr (1), Sachs (16), Young, Witebsky & Mohn (18), and Moullec & Diacono (13).

PRESENT INVESTIGATION

In 1948, when the blood group of a patient admitted to Pori General Hospital for duodenal ulcer was being determined, it was found that the cells reacted macroscopically like 0 cells, but only anti-B agglutinin was present in the serum. The blood was submitted to us for examination in 1951, and, in addition, we received samples from 14 subjects belonging to the patient's family. As Gammelgaard's publication on the basis of division of the weak A groups was not known to us at that time, the methods recommended by Fischer and Hahn were employed in the examination: agglutination, absorption and elution. Furthermore, the reaction of the cells to the extract prepared from a fungus (Psilocybe spadicea) (2) was studied. In the following, the samples belonging to the weak A blood group will be indicated by A_z.

Agglutination. — The experiment was carried out at room temperature (+ 20° C), both according to the slide method and in small test tubes (0.4 × 5 cm). The red cells of the persons belonging to the A_z group produced very similar reactions. Slide studies with B and O sera never gave a macroscopically visible, distinct agglutination. Test tube results with several different B sera (1 hour at 20°C) gave microscopic readings varying from negative to (+), while the A_2B cells employed as controls gave +++ — ++ reactions.[1] As a rule, O sera gave a stronger reaction with A_z cells

[1] Explanation of symbols:
+++ complete agglutination
++ incomplete agglutination
+ clumps just visible macroscopically
(+) clumps just visible microscopically
—? traces of agglutination by microscope
— microscopically homogeneous suspension
h hemolysis

TABLE 1

RESULTS OF THE ORDINARY BLOOD GROUP DETERMINATIONS OF 5 MEMBERS OF THE FAMILY

	Cells Examined					Contr. Cells	
	Su	JL	VK	HH	UK	A_1	B
A-serum	—	+++	+++	+++	—?	—	+++
B-serum	—	—?	—	—	—	+++	—

	Sera Examined				
	Su	JL	VK	HH	UK
A_1-cells	—	+	—	—?	+++
A_2-cells	—	—	—	—	++
B-cells	+++	—	—	—	++
Belongs to blood group	A_z	A_zB	A_zB	A_zB	O

than did B sera. Table 1 gives the results of the blood group determination of 5 persons examined simultaneously (test tube method). The identification symbols Su, JL, VK, HH and UK will be employed for the samples in the following.

Absorption. — A known B serum was absorbed by 1/1 Vol. of the examined cells as follows: 5 drops of serum and 5 drops of the cells to be examined were allowed to stand for 1 hour at room temperature, the tubes being shaken occasionally. The tubes were centrifuged at 3000 RPM and the supernatant titrated against A_1 and A_2 cells. The results are given in Table 2. (The same persons as in Table 1).

The cells of one of our A_z persons were also examined by comparing their absorption capacity with A_2B cells. The results are given in Table 3.

Elution. — 3 cc of inactivated B serum were absorbed by 3 drops of cells, centrifuged, the cells washed three times with saline solution, and elution was effected in 0.4 cc of saline solution at 56° C in the way described by Landsteiner and Miller (11). The results for the 5 blood samples included in Tables 1 and 2 are given in Table 4.

In addition A_z cells did not agglutinate at room temperature (20°C) or in the refrigerator (2—4°C) with agglutinin elutions obtained from these cells themselves.

TABLE 2

A COMPARISON OF THE ABSORPTION CAPACITY OF THE CELLS TO BE EXAMINED WITH KNOWN A_1 AND A_2 CELLS

		Tested against	Dilution								
			1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
Unabsorbed B-serum[1]		A_1	+++	+++	++	++	+	+	(+)	(+)	—
		A_2	+++	+++	++	++	+	(+)	—	—	—
B-serum absorbed by	1/1 Vol. of A_1 cells	A_1	—	—	—	—	—	—	—	—	—
		A_2	—	—	—	—	—	—	—	—	—
	1/1 Vol. of A_2 cells	A_1	++	++	(+)	—	—	—	—	—	—
		A_2	—	—	—	—	—	—	—	—	—
	1/1 Vol. of Su cells (A_Z)	A_1	+++	+++	++	++	(+)	(+)	(+)	—	—
		A_2	+++	++	++	++	(+)	(+)	—	—	—
	1/1 Vol. of JL cells (A_ZB)	A_1	+++	+++	++	++	+	(+)	—	—	—
		A_2	+++	+++	+++	++	+	(+)	—	—	—
	1/1 Vol. of VK cells (A_ZB)	A_1	+++	++	++	++	+	(+)	—	—	—
		A_2	+++	++	++	++	+	(+)	—	—	—
	1/1 Vol. of HH cells (A_ZB)	A_1	+++	+++	++	+	+	(+)	(+)	—	—
		A_2	+++	+++	++	++	(+)	(+)	—	—	—
	1/1 Vol. of UK cells (O)	A_1	+++	++	++	++	++	+	(+)	(+)	—
		A_2	+++	+++	++	++	+	(+)	—	—	—

[1] The A_Z cells were not agglutinated by the B-serum.

TABLE 3.

A COMPARISON OF THE ABSORPTION CAPACITY OF A_Z CELLS WITH KNOWN A_1, A_2B AND O CELLS

		Tested against	Dilution								
			1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
Unabsorbed B-serum[1]		A_1	+++	+++	+++	+++	+++	+++	++	(+)	—
		A_2B	+++	++	++	++	+	+	(+)	—	—
B-serum absorbed by	1/1 Vol. of A_1 cells	A_1	—	—	—	—	—	—	—	—	—
		A_2B	—	—	—	—	—	—	—	—	—
	1/1 Vol. of A_2B cells	A_1	+++	++	++	++	+	—	—	—	—
		A_2B	—	—	—	—	—	—	—	—	—
	1/1 Vol. of A_Z cells	A_1	+++	+++	+++	+++	++	++	(+)	—	—
		A_2B	+	(+)	—	—	—	—	—	—	—
	1/1 Vol. of O cells	A_1	+++	+++	+++	+++	++	++	++	+	—
		A_2B	+++	++	++	++	+	+	(+)	—	—

[1] The A_Z cells were not agglutinated by the B-serum.

TABLE 4

RESULTS OF THE ELUTION EXPERIMENT

Agglutinin solution Eluated from	Tested against	Dilution							
		1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
A_1 cells	A_1	+	+	(+)	—	—	—	—	—
	A_2	—	—	—	—	—	—	—	—
A_2 cells	A_1	++	++	+	(+)	—	—	—	—
	A_2	++	+	(+)	(+)	—	—	—	—
Su cells (A_Z)	A_1	++	++	+	(+)	—	—	—	—
	A_2	++	++	+	+	—	—	—	—
JL cells (A_ZB) ..	A_1	++	++	+	(+)	—	—	—	—
	A_2	++	++	+	(+)	—	—	—	—
VK cells (A_ZB) ..	A_1	++	++	+	(+)	—	—	—	—
	A_2	++	++	+	(+)	—	—	—	—
HH cells (A_ZB) ..	A_1	+++	++	+	(+)	—	—	—	—
	A_2	++	++	+	(+)	(+)	—	—	—
UK cells (O) ..	A_1	—	—	—	—	—	—	—	—
	A_2	—	—	—	—	—	—	—	—

EXPERIMENTS WITH PSILOCYBE SPADICEA EXTRACT

When studying the ability of fungal extracts to agglutinate the different human red cells (2), certain fungi were found capable of agglutinating A_1, A_2 and B cells with a considerably higher titre than O cells. The agglutinins were of the panagglutinin type, and absorption by any of the cells reduced the titre against the other cells as well. The titre of the extract prepared from Psilocybe spadicea fungus was·

	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
A_1	+++	+++	+++	+++	+++	+++	(+)	—?
A_2	*h*	+++	+++	++	+	(+)	—?	—
B	+++	+++	+++	+++	++	(+)	—	—
O	*h*	+	—	—	—	—	—	—

In the course of five months' storage (at +2° C) the reaction ability of the extract was so reduced that O cells no longer

TABLE 5

TITRE OF 10% SALINE SOLUTION OF THE FUNGUS PSILOCYBE SPADICEA BEFORE AND AFTER ABSORPTION BY A_1, A_2, Az AND O CELLS

Saline Solution of *Psilocybe spadicea*		Tested against	Dilution					
			1/1	1/2	1/4	1/8	1/16	1/32
Unabsorbed		A_1	+++	+++	++	+	—?	—
		A_2	++	(+)	—?	—	—	—
		A_z	—	—	—	—	—	—
		O	—	—	—	—	—	—
Absorbed by	1/1 Vol. of A_1 cells	A_1	—	—	—	—	—	—
		A_2	—	—	—	—	—	—
	1/1 Vol. of A_2 cells	A_1	—	—	—	—	—	—
		A_2	—	—	—	—	—	—
	1/1 Vol. of A_z cells	A_1	+	(+)	—?	—	—	—
		A_2	—?	—	—	—	—	—
	1/1 Vol. of O cells	A_1	+	—?	—	—	—	—
		A_2	—	—	—	—	—	—

agglutinated at all; the typical mode of reaction can be seen from the absorption tests carried out (Tab. 5).

On absorption by A_1 and A_2 cells (1/1 Vol.), the agglutinating capacity of the extract disappears completely, absorption by O cells leaves a very low titre against A_1 and A_2 cells. Absorption effected with the A_z cells gave a higher titre than the O cells. The result was controlled with the same cells, but unfortunately the extract did not suffice for the investigation of other samples of A_z type. The ability of A_z cells to absorb this phytagglutinin extract did not

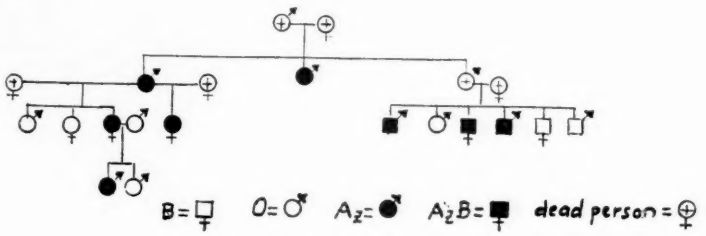


Fig. 1. — The heritage of the weak *a* gene.

therefore, at least in this case, come between A_2 and O, as might have been expected from the ordinary serum reactions, but was still weaker than that of O cells.

Genetic Investigation. — The heritage of the weak A gene in the family studied is shown in Fig. 1.

In the material studied the gene, as can be seen, has been inherited according to Mendel's law. The other ABO as well as MN and Rh agglutinogens present in the red cells of the A_z persons studied reacted normally with undiluted test sera.

DISCUSSION

A comparison of the present results with the reactions described by Gammelgaard as typical of the different groups shows that the cells, in their absorption capacity, are reminiscent of the A_5 group. In agglutinability they are obviously inferior to it, for the cells did not agglutinate at all with several anti-A sera, although A_2B cells were agglutinated up to a dilution of 1/64, and the agglutination picture reported as typical of A_3 was never present. In elution the result obtained with A_z cells was the same as or slightly stronger than that obtained with A_2 cells. Anti-A agglutinins active at room temperature were present in 4 out of the 8 studied samples belonging to the A_z group, in considerable strength in one only (JL), which again would fit in primarily with type A_4. Unfortunately we did not study the reaction of the cells with anti-O serum or the A-antigen content of the saliva, which might have decided the location of the group in Gammelgaard's system. However, it seems as if the blood type described by us might be some solitary mutation of the A-gene, not included in Gammelgaard's division, like the A_6 of Jonsson and Fast.

SUMMARY

A description has been given of the occurrence of a weak A blood group in a family. In its reactions, the group complied primarily with the A_5 group described by Gammelgaard. The genetic study of 15 persons reveals no deviations from Mendel's law. A reaction of blood cells to the extract prepared from a fungus (Psilocybe spadicea) is described. Our weak A cells seem to be weaker absorbers of agglutinins present in an extract of Psilocybe spadicea than A_1, A_2 and O cells.

REFERENCES:

1. Dahr, P.: Ztschr. f. Immunitätsforsch. u. exper. Therap. 1943: 102:13.
2. Elo, J., Estola, E., and Malmström, N.: Ann. Med. Exper. Biol. Fenniae 1951:*29:*297.
3. Fischer, W., and Hahn, F.: Ztschr. f. Immunitätsforsch. u. exper. Therap. 1935:*84:*177.
4. Friedenreich, V.: Ibid. 1936:*89:*409.
5. Gammelgaard, A.: Om sjældne, svage A-receptorer hos mennesket, Arnold Busck, Copenhagen 1942.
6. Gammelgaard, A., and Marcussen, P. V.: Ztschr. f. Immunitätsforsch. u. exper. Therap. 1940:*98:*411.
7. Hartmann, O., Hadland, K., and Lundevall, J. V.: Nord. Med. 1941:*10:*1136.
8. Hirszfeld, L., and Amzel, R.: Rev. d'immunol. 1940:*6:*31.
9. Jonsson, B., and Fast, K.: Acta Path. et Microbiol. Scandinav. 1948:*25:*649.
10. Kammann, E. O.: Ztschr. f. Immunitätsforsch. u. exper. Therap. 1942:*101:*289.
11. Landsteiner, K., and Miller: J. Exper. Med. 1925:*42:*853.
12. Morawiecki, J.: Schweiz. med. Wchnschr. 1941:*71:*.110
13. Moullec, J., and Diacono, G.: Rev. d'hémat. 1950:Tome *5:*318.
14. Moureau, P.: Acta Biol. Belg. 1943:*3:*17.
15. Moureau, P.: Ibid. 1943:*3:*22.
16. Sachs, H.: J. Path. & Bact. 1943:*55:*109.
17. Wiener, A. S., and Silverman, I. J.: Am. J. Clin. Path. 1941:*11:*45.
18. Young, L. E., Witebsky, E., and Mohn, J. F.: J. Immunol. 1945: *51:*111.

FROM THE HOSPITAL OF CONTAGIOUS DISEASES, HELSINKI, AND FROM THE DEPARTMENT OF FORENSIC MEDICINE, UNIVERSITY OF HELSINKI

THALLOUS SULPHATE POISONING

TWO CASES OF NON-FATAL ACCIDENTAL POISONING FROM PEAS USED FOR DESTROYING TAME PIGEONS

by

SIRKKA ISOTALO and A. R. ALHA

(Received for publication November 23, 1951)

On April 10—11, 1951, two little boys who had eaten peas soaked in thallous sulphate on April 2, were admitted to the Hospital of Contagious Diseases, with symptoms evidently pointing to a poisoning.

After the wars a special method of destroying pigeons by means of peas soaked in thallium was adopted in Helsinki. Heat-dried peas were allowed to stand for 2 days in a two per cent solution of Tl_2SO_4. The poisoning was carried out by the Municipal Sanitary Officer by scattering the peas where the birds usually gathered. He took care that any remaining peas were destroyed, and did this successfully for years without any accident. In the case now under consideration the Sanitary Officer who had recently taken up the post, happened to leave the place without collecting the superfluous peas, and the boys who had been watching managed to pick some up.

A number of cases of thallium poisoning have been described in the literature.

They have occurred when thallium has been improperly used in medicine, e.g. in the treatment of syphilis and tuberculosis, against which diseases thallous salts were used in the 1890'ies (9), and in thallium epilation used in the present century in treating skin diseases (1). The most numerous cases of poisoning were published in the 1930'ies (11) when I. G. Farbenindustrie's rat poison Zelio (which contains thallous sulphate) was widely used and gave rise to accidents and also to suicides and murders. In America, France, and England a popular cosmetic cream (Coremlucreme) con-

taining thallous acetate and used to remove superfluous hair caused a number of milder cases of poisoning (10, 11). There have also been cases of industrial poisoning (1) and food poisoning (10). In America, for instance, there had been 778 cases of thallium poisoning before 1934 (10), 46 of which were fatal.

Since 1935 altogether 8 cases of poisoning (2, 4, 5, 6, 7, 13), due to rat poison, have appeared in the Scandinavian literature.

In the Finnish literature we have only Vuori's case (15) in 1941, when rat poison had been used to provoke a miscarriage.

We have found no case in the literature of thallium poisoning occurring in the way just described, and since single cases of poisoning are continuously being published, we feel justified in describing this one.

CASE REPORTS

Case 1 (Hosp. Rec. No. 2173/51). — A working man's son, aged five years and a half, previously healthy. He ate an unascertained quantity of thallium peas on April 2, 1951, as was found later. There was abdominal pain on Apr. 3, vomiting and diarrhea, but no fever. On Apr. 5 he complained of pain in the legs. The amount of urine decreased. As the pain increased and the general condition worsened, a doctor was sent for on Apr. 11. He made arrangements for hospital treatment.

Case 2 (Hosp. Rec. No. 2156/51). — A stoker's son, aged 5, previously healthy apart from continuous enuresia and an intermittent stammer. He ate peas as the patient in Case 1, but evidently a smaller amount. On Apr. 5 his lips swelled and began to crack, the mouth felt dry. On Apr. 8 there was pain in the legs and he walked stiffly. On Apr. 9 he vomited, had still more difficulty in walking and had to support himself. There was no elevation in temperature and no diarrhea. On Apr. 9 a doctor was called, and since the patient's condition was still worse, he was sent to hospital on Apr. 10.

Clinical Reports. — See Table 1.

FORENSIC-CHEMICAL INVESTIGATION

Urine and excrements of both patients, as well as thallium peas, were examined chemically at the Department of Forensic Chemistry.

Urine and Excrements, taken on April 11—12, 51, were analysed by Fresenius-Babo's method (8) and revealed Tl by flame reaction and in the form of iodide, sulphide, and chromate. The Tl reactions were obviously stronger in Case 1 than in Case 2.

On April 13, peas found that day on the spot were sent to the Department as samples. Tl was discovered qualitatively as above, and a biological experiment was made on a white mouse; the mouse died within about 24 hours.

TABLE 1

	Date	General Condition, Weight, Height, Muscles, Skin, Mucous Membranes, Hair and Nails. Temperature	Digestive Organs and Kidneys
Case 1	11.4.	Very tired, pale. Weight 17 kg. Height 109 cm. Scanty subcutis. Cracked lips. The corners of the mouth Cracked. The conjunctivas strongly injected. Hair and nails 0. Rectal temp. 37° C.	Tongue moist, furred. Severe foetor. Mucous membrane of the mouth 0, pharynx 0. Abdomen, liver and spleen on palpation 0. No tenderness to tapping on kidneys. Excrement dark, loose, slimy, bad-smelling.
	14.4.	General condition worse. Lips and corners of the mouth have improved. Rectal temp. 37° C.	Frequent vomiting. 2—3 loose stools a day. Urine abundant. Wets his bed.
	18.4.	Very tired. Neither eats nor drinks. Muscles of the extremities atrophic. Hair falls profusely. Rect. temp. 36.8° C.	Frequent vomiting. No diarrhea. Polyuria and bedwetting.
	24.4.	Tired. Appears cachectic. Severe, purulent conjunctivitis. Purulent scab on eyelids, corners of the mouth and lips. Red, peeling patches on the knees, hips and forehead. Hair comes out in large tufts. Rectal temp. 37.2° C.	No vomiting. No diarrhea. Polyuria continues.
	2.5.	Livelier. Skin healed, hair continues to come out. Rectal temp. 36.8° C.	No stomach symptoms. Amount of urine normal.
	25.5.	Much livelier. Appetite good. Weight 15.8 kg. Has lost all hair from head and eyebrows. Eyelashes saw-edged. Down, about 2 mm long, covers the head. Rectal temp. 37.2° C.	Occasional bed-wetting.
	16.6. Sent home	General condition still better. Weight 17.1 kg. About ½ cm of thick hair. Skin healthy. Horizontal white stripes on finger nails. Rectal temp. 37° C.	No symptoms.

Organs of Blood Circulation	Nervous System	Laboratory Findings
Heart on auscultation 0. Pulse freq. 106/min. Ecg 0.	Fully conscious. No rigidity of neck or back. Stands and walks faultlessly. Romberg—. No paresis. Cerebral nerves 0. Reflexes normal. No disturbances of sensibility.	Blood picture normal. Serum calcium 14.8 mg%. Urine: Turbid, alb. +, sed. erythr. 1—3, leukoc. 8—12, granular casts 0—1, bact. —.
Heart on ausc. 0. Pulse freq. 100/min.	Speech confused. Complains he cannot see. Eye fundi 0. Cerebral nerves 0. No paresis. Reflexes ordinary	Urine: Alb. + (slightly), sed. 0.
Heart on ausc. 0. Pulse freq. 110/min. RR 120	Confused at times, sometimes replies to questions. Says he can see. Unable to sit up. No paresis. Cerebral nerves 0. Reflexes normal.	Urine 0.
Heart on ausc. 0. Pulse freq. 110/min. RR 130. Ecg 0.	Confused. Very restless. Roars like an animal. Does not speak. Waves his limbs with spasmodic and choreatic movements. No paresis. Reflexes normal.	Blood picture normal. Ser. protein fractions: Tot. prot. 6.2%, alb. 3.9%, glob. 2.3%. Sed. rate 1 mm. Icteric index 1: 14. Takata—. Non protein N 21.1 mg%. Sternal puncture normal. Liquor 0. Urine 0.
Heart 0. Pulse freq. 98/min. Ecg 0.	Speaks a few words, does not answer to questions. Can turn himself in bed, cannot sit. Continued muscular spasms, trembling and choreatic movements.	Icteric index 1: 10. Takata—. Sed. rate 10 mm. Urine 0.
Heart 0. Pulse freq. 92/min. RR 100.	Fully conscious. Replies to questions. Sits; walks when supported, with difficulty, dragging his feet. Still marked trembling and choreatic movements.	Hb 68/75. Liquor 0. Urine: Alb—.
Heart 0.	Fully conscious. Stands a while without support although with trembling of the whole body. Walks with support. No paresis. Reflexes normal.	Hb 68/75. Urine: Alb—.

	Date	General Condition, Weight, Height, Muscles, Skin, Mucous Membranes, Hair and Nails. Temperature	Digestive Organs and Kidneys
Case 1	20.8.	Has been well at home. Weight 19.5 kg. Height 110 cm. Skin healthy. Good hair, ab. 3 cm. Still stripes on the nails. Eyebrows normal.	No symptoms.
Case 2	11.4.	Tired. Weight 16.7 kg. Height 108 cm. Scanty subcutis. No skin trouble. Healthy conjunctivas. Rectal temp. 37.6° C.	Tongue moist, no coating. Mucous membrane of mouth 0, pharynx 0. Abdomen, liver and spleen on palpation 0. Excrements loose, foul smelling.
	18.4.	Tired. Neither eats nor drinks. Has lost weight. Reduced muscular elasticity. Lids reddened, swollen. Hair comes out profusely. Rectal temp. 36.8° C.	Frequent womiting on 2 days. No diarrhea. Polyuria, bed-wetting.
	24.4.	Tired. Eats better. Skin and mucous membranes healthy. Rectal temp. 37.2° C.	No vomiting. No diarrhea. Polyuria and bed-wetting.
	2.5.	General condition much better. Hair falls out in large tufts. Rectal temp. 36.6° C.	No stomach symptoms. Bed-wetting.
	25.5.	Has put on weight. Head and eyebrows quite bald. Two mm of down on the head. Rectal temp. 36.8° C.	Still wets his bead. Urine amount normal.
	16.6. Sent home	General condition good. Weight 16 kg. Skin healthy. Thick hair, ab. ½ cm. Nails normal. Rectal temp. 36.8° C	Wets his bed sometimes, at night especially.
	20.8.	Has been well at home. Weight 17.4 kg. Height 109 cm. About 3 cm of good hair. Eyebrows normal.	Sometimes wets his bed at night.

Organs of Blood Circulation	Nervous System	Laboratory Findings
Heart on ausc. 0.	Walks without support but atactically. Still trembling of the upper limbs. Romberg—. Finger-nose test uncertain. Reflexes normal.	Hb 71/78.
Heart on ausc. 0. Pulse freq. 125/min. Ecg 0.	Fully conscious. Walks a little unsteadily. No rigidity in neck or back. No paresis. Cerebral nerves 0. Reflexes normal. No disturbances of sensibility.	Blood picture normal. Ser. calcium 15.3 mg%. Urine: Alb + (slightly), sed. erythroc. 2—5, leucoc. 5—8, bact—.
Heart 0. Pulse freq. 120/min. RR 140.	Fully conscious. Answers questions. Cannot sit up. Cerebral nerves normal. No paresis. Reflexes normal.	Urine 0.
Heart on ausc. 0. Pulse freq. 120/min. RR 120. Ecg 0.	Fully conscious. Talks. Lies prostrate, cannot sit up. No paresis.	Blood picture normal. Ser. protein fract.: Tot. prot. 7.2%, alb. 4.0%, glob. 3.2%. Sed. rate 5 mm. Icteric index 1: 11. Takata—. Nonprotein N 23.3 mg%. Liquor 0. Urine 0.
Pulse freq. 100/min.	Markedly livelier. Grasps objects. Nystagmus in the eyes. Squints inward alternately with both eyes. Eye fundi 0. Cerebral nerves otherwise normal.	Icteric index 1: 4. Takata—. Sed. rate 30 mm. Urine 0.
Pulse freq. 95/min. RR 100. Ecg 0.	Walks with support. Trembling in extremities. Eyes 0.	Hb 65/72. Sed. rate 28 mm. Liquor 0. Urine 0.
Pulse freq. 90/min.	Walks staggeringly without support. Romberg+. Reflexes normal.	Hb 66/73. Sed. rate 20.
Heart on ausc. 0. Pulse freq. 80/min.	Walks freely. Some unsteadiness when turning round. Coarse tremor of the hands. Romberg—. Finger-nose test uncertain.	Hb 76/84.

Excrement and urine were taken for examination in both cases on April 28. The quantity of Tl was determined from the specimens by Fridli's method (3). In Case 1 there were 8.4 mg Tl in 10 g of excrement and 1.45 mg Tl in 100 ml of urine, and in Case 2 6.2 mg Tl in 10 g of excrement and 0. 24 mg Tl in 100 ml of urine. There was again an obviously larger quantity of Tl in Case 1.

The cases illustrate the typical course of acute thallous salt poisoning (12, 14). On the two first days the patients had no symptoms, then local symptoms appeared on the spots touched by the poison. Resorptive symptoms appeared at the same time, the first of which was polyneuritic pain in the lower extremities. After 2—3 weeks the deterioration of the general condition characteristic of thallium poisoning reached its height and the resorptive symptoms were at maximum. The most obvious symptoms, such as skin troubles and loss of hair, pointed to disturbance of the endocrine-sympathetic system and there were strong symptoms traceable to the central nervous system. In our cases the symptoms which occur in protoplasm poisoning, indicating changes in the liver and kidneys and other internal organs, were slight: there was transient nephropathia and tachycardia, lasting one week only, a slightly increased icterus index and anemia of the normochromic type. The loss of weight should also be mentioned. Yet there was evidently no disturbance of growth.

After 6—7 weeks the patients' condition began to improve rapidly and they were dimissed after 11 weeks.

In the 5 month follow-ups the patients' health improved steadily, although Case 1, the severer of the two cases, still suffered from ataxia when walking and both children were timid and easily frightened following the nervous disturbances, and their sleep at night was broken.

The literature mentions no specific treatment for thallium poisoning, although BAL has been recommended. In the present cases sodium thiosulphate and vitamin B treatment could not be said to have had any definite effect.

SUMMARY

Two little boys aged 5 had eaten peas soaked in thallous sulphate meant for destroying tame pigeons. This was revealed from the report of the accident, in the forensic-chemical examination, and by the typical clinical features of thallium poisoning noted in the cases.

REFERENCES

1. Buschke, A.: Dermat Ztschr. 1938:*77:*186.
2. Carlgren, A.-E.: Acta Ped. 1950:*39:*438.
3. Fridli, R.: Dtsch. Ztschr. gerichtl. Med. 1930:*15:*478.
4. Gjertz, A.: Acta Med. Scand. 1935:*85:*531.
5. Hofman-Bang, A.: Ugeskr. f. Læger 1937:*99:*760.
6. Jakobsen, R.: Ugeskr. f. Læger 1939:*101:*1445.
7. Johnsson, S.: Sv. läkartidn. 1949:*46:*773.
8. Lieb, H.: Der gerichtlich-chemische Nachweis von Giften in: E. Abderhalden: Handbuch der biologischen Arbeitsmethoden. Abt. IV, Teil 12, S. 1471. Urban & Schwarzenberg, Berlin & Wien 1938.
9. Lucke, B., and Kolmer, J. A.: Arch. of Neurol. 1923:*10:*288.
10. Munch, J. C.: J.A.M.A. 1934:*102:*1929.
11. Oster, H.: Samml. v. Vergifts. fäll. 1940:*11:*55.
12. Reuter, F.: Methoden der forensischen Beurteilung von Vergiftungen in: E. Abderhalden: Handbuch der biologischen Arbeitsmethoden. Abt. IV, Teil 12, S. 1075, Urban & Schwarzenberg, Berlin & Wien 1938.
13. Rignér, K.-G.: Sv. läkartidn. 1950:*47:*2095.
14. Sollmann, T.: A Manual of Pharmacology. W. B. Sounders Co, Philadelphia & London 1950.
15. Vuori, A. K.: Nord. Med. 1941:3565.

FROM THE CHILDREN'S CLINIC AND THE DEPARTMENT OF PHYSIOLOGY,
UNIVERSITY OF HELSINKI

RELEASE OF ANTIDIURETIC HORMONE DURING NURSING IN DOG

by

H. KALLIALA, M. J. KARVONEN, and V. LEPPÄNEN

(Received for publication December 11, 1951)

Indirect evidence has been presented by several workers suggesting the release of posterior pituitary hormone during the act of nursing (3, 5, 9, 13 14, 19, 22, 25, 28, 29, 30, 33, 34, 35). (Terminology: 7). The effects due to the release of the hormone — the ejection of milk and uterine contractions (21) — have generally been ascribed to the oxytocic fraction of the hormone, but other fractions may also play a rôle in this response (34).

However, it is questionable whether any of the known effects of the posterior pituitary hormone is physiologically produced without the concomitant actions of the other fractions (6, 10, 16, 38). It has even been claimed that the fractionation of the posterior pituitary hormone is a mere artifact (1, 2, 32, 36, 37). Thus, it is reasonable to expect that the antidiuretic action of posterior pituitary hormone is observable during the act of nursing, if a suitable experimental arrangement is employed. Such a finding would strongly support the theory of the release of posterior pituitary hormone during nursing, even if also this observation is no direct proof of that theory. A direct proof may be obtained only by the means of hormone assays on blood or urine.

Antidiuretic responses have in fact been observed in connection with milking in cows (26, 27). Two of the present writers (17) have also demonstrated antidiuresis during nursing in human

subjects, but the response was not found in all mothers. Therefore, the study was extended to cover another species, the dog, and a slightly different method of experimentation was used. In order to gain a more comprehensive picture of the changes produced, the determination of the urinary sodium excretion was included in the programme, and an indirect assay of the amount of hormone released was attempted.

MATERIAL AND METHODS

The experiments were performed on three unanesthetized laboratory-bred mongrel bitches, Corporal, Jesse, and Pelle. Corporal and Pelle were sociable dogs, whereas Jesse was restless and had to be kept in a cage, since it evidently was not an accepted member of the laboratory dog community. All the bitches were trained before and during pregnancy to the administration of water by gavage and to the collection of urine through a catheter. After the delivery, all but one of the puppies of Corporal were killed, and the mother was allowed to rear the remaining one. Jesse was left with all of its four puppies; two of them died and the remaining two had to be given soon to another lactating dog. Therefore, the number of experiments on this dog had to be cut to less than intended. Pelle nursed three puppies.

The dogs were kept fasting the night preceding the experiments, but water or milk was allowed ad libitum. In the morning, the dogs were given 30 ml. of tepid water per kg of body weight, by gavage, in order to bring them as close to a standard state of hydration as possible. They were catheterized (12) after about an hour, and exactly 90 minutes after the first gavage a second dose of water was given. After this, urine was collected every 10 or 15 minutes, its volume was noted, and its sodium concentration was analysed, by using a flame photometer. In the course of the work, two flame photometers were used (15, 18). The puppies were kept separate from their mother, until they were allowed to start suckling. The time of suckling varied somewhat in the individual experiments. In the control experiments, the procedure was the same, except for the omission of the act of suckling. During the period of lactation, suckling experiments and controls were performed alternately. After weaning, assay experiments by administering Pituitrin (Parke &

Davis) intravenously were performed on the same dogs. All the experiments were made in a calm room, and an attempt was made to avoid all disturbance to the animal.

On Corporal, three control experiments were performed during pregnancy, five during lactation, and two after the puppy had been weaned. The number of the suckling experiments was three, and the number of the pituitrin experiments was similarly three. The delivery occurred 16/12-1949.

On Jesse, similarly three control experiments were made during pregnancy, two during lactation, and two after weaning. The number of the suckling experiments was only two; in one of them, the young started the suckling twice. The suckling had to be discontinued prematurely because of obviously insufficient milk production. The failure was perhaps ascribable to the aggressive mood of the mother: it was fighting all the time with its companions through the meshes of the cage (see: 20). Two assay experiments with Pituitrin were made on this dog. The delivery occurred 9/11-1950.

The number of control experiments on Pelle was 5; of these 2 were made during pregnancy and 3 during the period of lactation. 4 suckling experiments and 3 assay experiments with Pituitrin were performed. The delivery occurred 4/4-1951.

Owing to the amount of urine contained in the catheter and in the urinary tract, changes in the excretion of Na do not immediately manifest themselves in the samples collected. For a temporal analysis of the sodium excretion values, it is important to know the volume of this »dead space», and also, whether there is any significant mixing of urine in the urinary tract or whether it travels more or less as a column.

For this purpose, 1 or 2 ml. of 1 per cent methylene blue solution was injected intravenously to one of the dogs, Jesse, and the collection of urine was started immediately. In one experiment at a high rate of urine flow (appr. 4 ml. per min.), the blue colour appeared in the urine after 5 ml. had been collected. In successive 5 ml samples of urine after the injection, the relative concentration of the dye varied as 0-100-81-68-44, as measured with a 'Lumetron' photoelectric colorimeter. In a corresponding experiment at a low rate of urine flow (appr. 0.4 ml per min.), the blue colour appeared after 2 ml had been collected, and the intensity of the colour in successive 2 ml samples varied as 0-20-44-98-100-23-20-17-18. Thus, the maximum concentration of the dye was reached during the interval 5—10 ml and 6—10 ml, respectively.

It may be concluded that the 'dead space' of the urinary tract plus catheter is not more than approximately 5 to 6 ml, and at a

high rate of urine flow, apparently less mixing occurs than at low rates. Thus, a significant time lag in the sodium excretion values as compared to those of the urine flow occurs only at low rates of urine flow: e.g. at a rate of 0.5 ml per min., a 10 min. sample entirely represents the preceding period, but at a rate of 5 ml per min., the lag is only 1 minute.

RESULTS

Urine Flow. — In each dog, the *control* experiments outside the period of lactation showed a fairly regular course of diuresis. Because of individual characteristics in the excretion curves, the material was not treated as a whole, but separately for each dog.

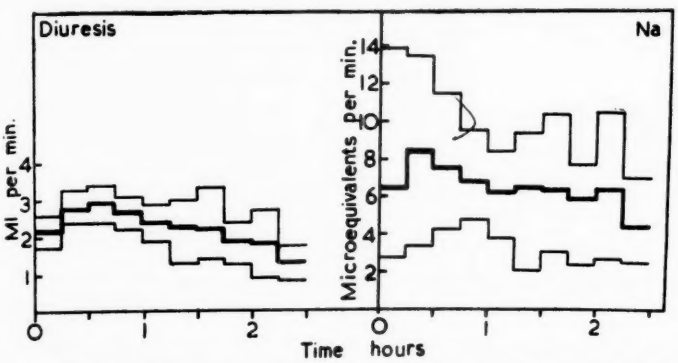
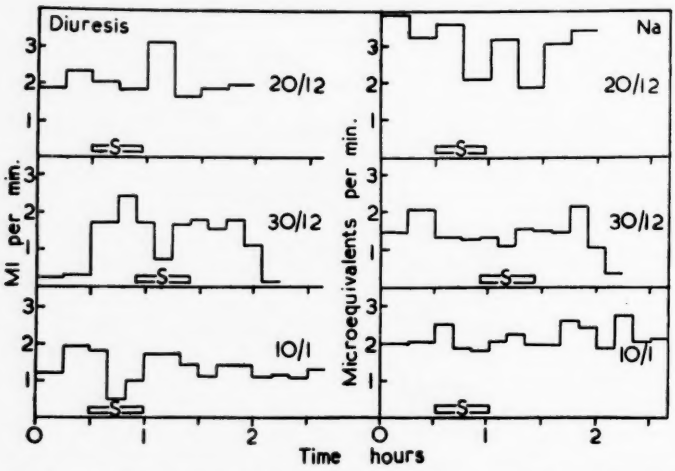


Fig. 1. — The average course (thick line) and the range of the variation (thin lines) of urine flow (left) and sodium excretion (right) in the control experiments on dog Corporal outside the period of lactation. The second dose of water was administered at time zero.

Fig. 1 shows the average course and the range af variation for diuresis in Corporal in control experiments outside the period of lactation.

In the *suckling* experiments on Corporal, the puppy was taken to the teat 30, 55, and 30 min. after the administration of the second dose of water, respectively. Figures 2 to 4 show the course of diuresis in each of these experiments. In the first and third of them, the rise of urine flow which otherwise would have been expected at that time was converted to a fall, when the puppy started suckling. A similar antidiuresis occurred also in the second



Figs. 2—4. — Urine flow (left) and sodium excretion (right) in three suckling experiments on dog Corporal. 'S' denotes the time of suckling.

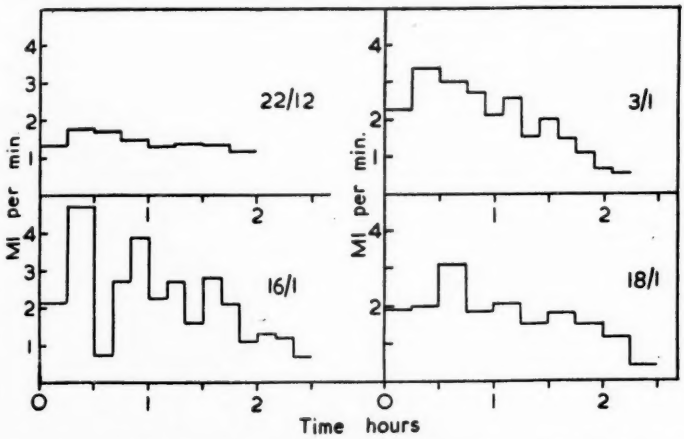
experiment. The antidiuretic response passed again within approximately ½ hr. after the beginning of the suckling.

In Jesse and Pelle the experiments gave also a distinct antidiuresis on suckling in every experiment. In the first experiment on Jesse, the puppies were allowed to start feeding 30 min. after the second dose of water, and they started it again spontaneously 65 min. later. At each time, a steep fall, similar to that shown in Fig. 3, ensued.

In Corporal and Jesse the minimum was never reached immediately after the onset of the suckling, but in the samples collected 20 min. (4 times) or 30 min. (twice) after the start.

However, in Pelle the lowest rate of urine flow was observed in three experiments already during the first ten minutes of suckling and only in one experiment in the second ten-minute period.

In the *control experiments* made on Corporal *during lactation* an interesting development was observed (Figs. 5—8). The first two experiments did not show any unusual features. However, in the remaining three experiments a more or less pronounced antidiuretic response was observed at about the same time as in the suckling experiments. In one of these experiments the antidiuretic response could be attributed to the restlessness of the animal, but in the remaining two, no obvious reason for the inhibition of diuresis



Figs. 5—8. — Urine flow in four control experiments on dog Corporal during lactation. An 'anomalous' antidiuretic response is observed in the experiment of 16/1 and, less distinctly, in that of 18/1.

was noticed. Because of this irregularity, assay experiments were postponed to a period when the control experiments had again assumed their normal course.

In Jesse and Pelle, the controls showed a regular course throughout the period of experimentation.

In order to get an approximate estimate of the amount of pituitary antidiuretic hormone which is required to effect similar antidiuretic responses as observed, three *assay* experiments were made on Corporal, two on Jesse, and three on Pelle.

The antidiuretic response of the suckling experiments could be closely imitated by injecting intravenously a suitable dose of Pituitrin. Fig. 9 shows an example of the close qualitative similarity between such antidiuretic responses in Pelle.

The percentage reduction of the urine flow from its pre-injection level was employed as the basis of the biological standardisation (23). The results of the assay experiments, as well as the corresponding figures for the experiments in which antidiuresis was observed, are shown in Table I. It is observed that the response seen in the suckling experiments was of the same order as that produced by 0.01 to 0.5 milliunits (mU) of Pituitrin. The same is true of the 'anomalous' control experiments on Corporal (Figs. 5—8).

Sodium. — In the *control* experiments, the excretion of sodium

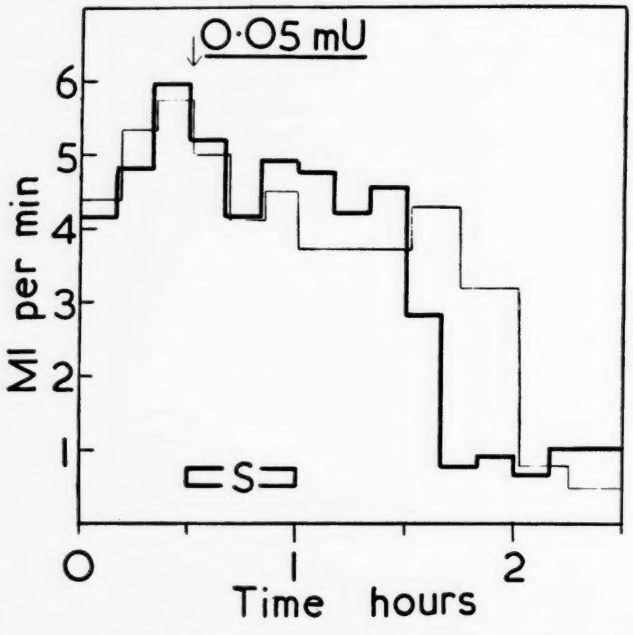


Fig. 9. — Urine flow in a suckling experiment (thick line) and in an assay experiment (thin line) on dog Pelle. An arrow denotes the time of the injection, and 'S' the time of the suckling.

showed a relatively much greater variation than the urine flow (Fig. 1). This was to be expected, as no attempt was made to standardize the experimental conditions by the administration of a standard dose of salt (22), similar to the prehydration dose of water. Again, individual differences obtained between the dogs, Jesse showing almost consistently smaller excretion values than Corporal, whereas the values of Pelle showed a wide range of variation, from 1.23 to 84.5 micro-equivalents per min.

The course of the excretion of sodium during the *suckling* experiments was less uniform than that of the urine flow, and also much more variable than the sodium excretion in the control experiments. An examination of the figures shows that in all dogs, the *excretion* of sodium increased immediately on the onset of suckling every time — except for two experiments on Pelle — independently, whether the urine flow was already falling (Corporal and Pelle), or whether its fall was delayed to the following 10 min.

TABLE I

ASSAY OF THE ANTIDIURETIC RESPONSE DURING NURSING IN THE TERMS OF THE ANTIDIURETIC EFFECT OF INTRAVENOUSLY INJECTED 'PITUITRIN'

		Pituitrin Miliunits	Reduction of Diuresis per cent
Corporal	Pituitrin	0.1	47
		0.5	88
		1.0	98
	Nursing 20/12	$<$ 0.1	21
	30/12	0.1—0.5	69
	10/1	0.1—0.5	74
	Control 16/1	$\sim$ 0.5	84
	18/1	$\sim$ 0.1	40
Jesse	Pituitrin	0.1	50
		0.5	96
	Nursing 15/11	0.1—0.5	71
	$15/11_{II}$	0.1—0.5*	58
	22/11	$\sim$ 0.1	53
Pelle	Pituitrin	0.01	0
		0.05	28
		0.2	37
	Nursing 10/4	0.05—0.2	30
	17/4	0.01—0.05	10
	24/4	0.01—0.05	15
	5/5	0.01—0.05	25

* This result is not strictly comparable with the others, as the suckling started as late as 95 min. after gavage. However, the urine flow was at that time still occurring at as high a rate as 3.15 ml per min., which is of the same order as the pre-nursing urine flow in the other experiments.

period (Jesse). In the two 'anomalous' experiments on Pelle, a rise in the sodium excretion was delayed to the following 10 min. period. With the decrease of the urine flow, a fall occurred also in the excretion of sodium in most experiments, although its *concentration* always increased. The subsidence of the antidiuretic response was regularly followed by a moderate increase in the sodium excretion (Figs. 2—4), most markedly in Jesse and Pelle.

In the 'anomalous' controls on Corporal during lactation, the sodium values showed no regular features.

In the *assay* experiments, the excretion of sodium experienced changes at least qualitatively similar to those seen in connection with suckling. After the injection of 0.01 to 0.5 mU Pituitrin, there

was either an immediate rise of the sodium excretion, in spite of a simultaneous drop in urine flow (Corporal and Pelle), or a slight fall in the excretion which, however, was proportionately less than the simultaneous fall in urine flow (Jesse).

With the antidiuresis, which was reached in the samples collected from 10 to 30 minutes after the injection, the excretion of Na also fell to relatively small values. The first sample collected during the subsidence of antidiuresis showed a rise in the excretion of Na. This rise is, at least partly, due to the 'dead space': approximately 5 ml of the aforementioned samples represents the concentrated urine as excreted during the minimum urine flow.

DISCUSSION

The act of nursing produced an antidiuretic response in these experiments. A passing antidiuresis is known to result either from the release of the antidiuretic hormone of the posterior pituitary, or from hemodynamic changes. The registration of the arterial blood pressure under the conditions of the experiments was not feasible, but, nevertheless, the contribution of hemodynamic factors can be regarded as most unlikely for the following two reasons:

(1) The antidiuretic response was of a 'slow' type, typical of a release of the posterior pituitary hormone, instead of a 'rapid' antidiuresis due to the release of adrenaline or to other hemodynamic factors (24).

(2) The marked changes in the electrolyte excretion and concentration in urine which were observed are also characteristic effects of the posterior pituitary antidiuretic hormone, and not of a hemodynamically conditioned antidiuresis, in which the excretion of urine and of the electrolytes tend to parallel variation (24). Similar findings: antidiuresis with a concomitant rise in the chloride concentration of the urine have been reported by the Belgian workers in the lactating cow (26).

This evidence, in combination with all the previous work on the rôle of the posterior pituitary in nursing, brings the release of the antidiuretic hormone during the act of nursing as close to certainty as indirect evidence may ever be expected to do.

A small but definite absolute increase in the Na excretion

occurred in connection both with the physiological release of antidiuretic hormone and with the administration of small doses of Pituitrin. This increase may be ascribed to the release of physiological amounts of either the oxytocic component (8, 11) or of the pressor-antidiuretic fraction (4) of the posterior pituitary hormone (31).

An assay of the antidiuretic response in the terms of post-pituitary extract shows that the release is of the same order as that produced in an osmotic antidiuresis (39). That such a response, the conservation of water, is purposeful to the animal in preserving the balance of its *milieu interieur* in connection with the loss of hypotonic fluid, milk, has already been pointed out (17).

The 'anomalous' control experiments encountered with in the dog Corporal during lactation deserve special mention. This bitch evidently had 'good nursing characteristics', as judged from the growth of its young. It is known that the neurohormonal milk ejection reflex in cattle and also in man has a tendency to become conditioned to various circumstances (40). Similarly, it is quite possible that nursing in the experimental setup did become a conditioned reflex in this dog, which reflex, when once acquired, was not broken down by the alternating control experiments.

The results of the present work are in an agreement with the theory of a release of posterior pituitary hormone during the act of nursing and they show that the antidiuretic component of this hormone evidently is also secreted as a result of the milk ejection reflex in dog.

SUMMARY

1. The urine flow and the urinary sodium excretion of trained lactating bitches were studied in connection with the act of nursing after an administration of water.

2. Nursing produced an antidiuresis and a passing increase in the excretion of sodium, comparable to those produced by an intravenous injection of 0.01 to 0.5 milliunits of Pituitrin.

Acknowledgements. — The writers wish to thank Miss Eila Ala-Ketola for assistance in the experiments. Two of the writers (M. J. K. and V. L.) obtained a personal grant from 'Lastentautien Tutkimussäätiö'('Foundation for the Study of Children's Diseases').

REFERENCES

1. ABEL, J. J.: J. Pharmacol. 1939:*40:*139.
2. ABEL, J. J., and C. A. ROUILLER: J. Pharmacol. 1923:*20:*65.
3. ANDERSSON, B.: Acta Physiol. Scand. 1951:*23:*1, 8, 23.
4. ANSLOW, W. P., WESSON, L. G., BOLOMEY, A. A., and J. G. TAYLOR: Feder. Proc. 1948:*7:*3.
5. BRAUDE, R., and K. G. MITCHELL: Nature (Lond.) 1950:*165:*937.
6. COLLIN, R., and F. STUTINSKY: J. de Physiol. 1949:*41:*7.
7. COWIE, A. T., FOLLEY, S. J., CROSS, B. A., HARRIS, G. W., JACOBSOHN, D., and K. C. RICHARDSON: Nature (Lond.) 1951:*168:*421.
8. DICKER, S. E., and H. HELLER: J. Physiol. 1946:*104:*353.
9. ELY, F., and W. E. PETERSEN: J. Dairy Sci. 1941:*24:*211.
10. FISHER, C., RANSON, S. W., and H. W. MAGOUN: Am. J. Obst. Gynaec. 1938:*36:*1.
11. FRASER, A. M.: J. Physiol. 1942:*101:*236.
12. FRIBERG, O., KARVONEN, M. J., and V. LEPPÄNEN: Ann. Med. Exp. Biol. Fenn. 1950:*28:*144.
13. GOMEZ, E. T.: J. Dairy Sci. 1939:*22:*488.
14. GOMEZ, E. T.: J. Dairy Sci. 1940:*23:*537.
15. HALLMAN, N., and V. LEPPÄNEN: Suomen Kemistilehti 1949:B *22:*55.
16. HARRIS, G. W.: Physiol. Rev. 1948:*28:*139.
17. KALLIALA, H., and M. J. KARVONEN: Ann. Med. Exp. Biol. Fenn. 1951:*29:*233.
18. LEPPÄNEN, V., METTINEN, T., and O. FORSANDER: To be published shortly.
19. MACKENZIE, K.: Quart. J. Exp. Physiol. 1911:*4:*305.
20. NEWTON, M., and N. R. NEWTON: J. Pediatr. 1948:*33:*698.
21. NEWTON, N. R., and M. NEWTON: Pediatrics 1950:*5:*726.
22. O'CONNOR, W. J.: Quart. J. Exp. Physiol. 1950:*36:*21.
23. O'CONNOR, W. J., and E. B. VERNEY: Quart. J. Exp. Physiol. 1942:*31:* 393.
24. O'CONNOR, W. J., and E. B. VERNEY: Quart. J. Exp. Physiol. 1945:*33:* 77.
25. OTT, I., and J. C. SCOTT: Proc. Soc. Exp. Biol. Med. 1911:*8:*48.
26. PEETERS, G., and R. COUSSENS: Arch. Int. Pharmacodyn. 1950:*84:*209.
27. PEETERS, G., COUSSENS, R., BOUCKAERT, J. H., and W. OYAERT: Arch. Int. Pharmacodyn. 1949:*80:*355.
28. PEETERS, G., MASSART, L., and R. COUSSENS: Arch. Int. Pharmacodyn. 1947:*14:*151.
29. PETERSEN, W. E.: Proc. Soc. Exp. Biol. Med. 1942:*50:*298.
30. PETERSEN, W. E., and T. M. LUDWICK: Feder. Proc. 1942:*1:*66.
31. ROEMMELT, J. C., SARTORIUS, O. W., and R. F. PITTS: Am. J. Physiol. 1949:*159:*124.
32. ROSENFELD, M.: Bull. Johns Hopkins Hosp. 1940:*66:*398.
33. SCHÄFER, E. A.: Quart. J. Exp. Physiol. 1913:*6:*17.
34. TURNER, C. W., and W. D. COOPER: Endocrinology 1941:*29:*320.

35. Turner, C. W., and I. S. Slaughter: J. Dairy Sci. 1930:*13:*8.
36. Vaichulis, J. A.: J. Pharmacol. 1939:*66:*37.
37. Vaichulis, J. A.: Endocrinology 1943:*32:*361.
38. van Dyke, H. B., Chow, B. F., Creep, R. O., and A. Rothen: J. Pharmacol. 1942:*74:*190.
39. Verney, E. B.: Proc. Roy. Soc. B. 1947:*135:*25.
40. Waller, H. K.: Lancet 1943:*I:*69.

INSTITUT FÜR ARBEITSMEDIZIN UND -HYGIENE

Von

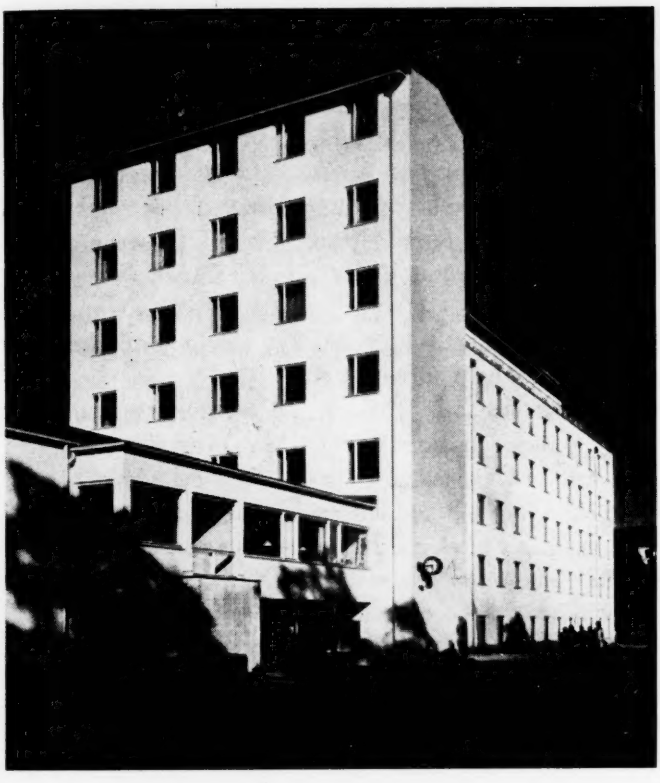
LEO NORO[1]

Das Institut für Arbeitsmedizin und -hygiene, das in dem Distrikt des Universitätskrankenhauses Meilahti in Helsinki seine Tätigkeit begonnen hat, hat im Vergleich zur Entwicklung dieses Arbeitsgebietes in Finnland nur eine kurze Vorgeschichte. Der Gedanke eines solchen Institutes tauchte erst im Jahre 1945 auf, als in die Verbindung der ersten medizinischen Universitätsklinik Anfang April eine Abteilung und Poliklinik gegründet wurde, die hauptsächlich für die Forschung und Pflege der zahlreichen Kohlengasfälle von jener Zeit bestimmt war. In demselben Jahre am 30. Mai wurde eine Stiftung für Unterstützung der arbeitsmedizinischen Forschung gebildet, in deren Kreise man es von Anfang an für nötig hielt, eine Forschungsinstitut des Gebietes zu errichten. In dem folgenden Jahre machte ein von dem Staatsrat ernanntes Komittee den Vorschlag, ein Institut auf staatliche Kosten zu bauen. Der Reichstag gab noch in demselben Jahre den ersten Zuschuss für den Beginn der Bauarbeiten. Der Grundstein des Instituts wurde im September 1948 gelegt. Vor allem wegen der zahlreichen Streiks sind die Bauarbeiten sehr verzögert worden, so dass das Institut erst im Frühling 1951 fertig wurde.

Das Institut für Arbeitsmedizin und -hygiene ist ein ungefähr 30,000 m³ fassender Baukomplex, dessen Projektierung durch den dreieckigen engen Bauplatz sehr schwer war. Von den Besonderheiten des Baus seien das Klimazimmer mit der Laufbahn, in dem die Feuchtigkeit und Temperatur innerhalb weiter Grenzen geregelt werden kann, und die Unterdruckkammer erwähnt.

Das Institut ist staatliches Eigentum, geht aber bis zum Ende des Jahres 1954 auf die Stiftung für Arbeitsmedizin über. In der Direktion der Stiftung sind 15 Mitglieder, von welchen der Staats-

[1] Med.Dr., Leiter des Instituts.



rat 8, der Zentralverein der Arbeitsgeber in Finnland 2, der Zentralverbund der finnischen Gewerkschaftsvereine 2, der Zentralverbund der Versicherungsanstalten, die Stadt Helsinki, und der Verein für die Abwehr der Unfälle (Tapaturmantorjuntayhdistys) je 1 ernennt.

Die Direktion der Stiftung gibt dem Institut einen Vorstand von 7 Personen, der unmittelbar für die Leitung des Instituts sorgt.

Die Ausgabe des Instituts ist in dem Reglement folgenderweise festgelegt:

1. arbeitshygienische und -medizinische Methoden zu erforschen und zu entwickeln, mit welchen die Gesundheit der Arbeiter geschützt und verbessert werden kann;

2. auf Wunsch der Behörden und Privatpersonen sowohl arbeitshygienische Untersuchungen auf den Arbeitsstellen als auch

andere Forschungsaufgaben auf dem Gebiete der Arbeitshygiene und der damit eng verbundene Gesundheitspflege auszuführen;

3. mit der arbeitshygienischen Sachkenntnis die staatlichen und kommunalen Behörden und Anstalten, die für die Hebung der Gesundheit der Arbeiter wirken, zu unterstützen.

4. nach Möglichkeit auf Wunsch Besprechungen über sozialmedizinische und arbeitshygienische Fragen mit Ärzten, technischen Funktionären, Sozialleitern, den Vertrauensmännern der Betriebe und anderen mit diesen vergleichbaren Personen durchzuführen;

5. als eine diagnostische Anstalt und ein klinisches Zentrum der Gewerbekrankheiten zu wirken;

6. verabredetermassen als ein Zentrum der Gesundheitsfürsorge einiger Betriebe, staatlichen Büros, Anstalten, Schulen und Lehranstalten in Helsinki zu fungieren, in dem Umfang, wie hierdurch die Forschungs- und Lehrtätigkeit der Anstalt gefördert wird.

7. kursusförmigen Unterricht in der Arbeitshygiene sowie Sozial- und Arbeitsmedizin zu geben;

8. zusammen mit den staatlichen Organen und interessierten Vereinen und Organisationen sozial- und arbeitshygienische Aufklärungsarbeit zu leisten;

9. die anderen Aufgaben auf dem Gebiete des Instituts zu besorgen, die die Direktion der Stiftung dem Institute vorlegt.

Das Institut besteht aus fünf Abteilungen:

1. die allgemeine Abteilung, deren hauptsächliche Aufgabe es ist, für die unmittelbare Verwaltung und Leitung, Ökonomie und Verbindungen, statistische Arbeiten und Untersuchungen, arbeits- und sozialhygienische Aufklärungsarbeit, sowie auch für die Aufgaben des Instituts, die zu keiner anderen Abteilungen hören, zu sorgen;

2. die medizinische Abteilung mit der Aufgabe, arbeitsmedizinische Forschung zu fördern, die poliklinische und klinische Tätigkeit zu überwachen, für die arbeitshygienischen Verhältnisse der Arbeitsplätze, Büros, Anstalten, Schulen usw., die als Versuchsfeld gewählt sind, zu sorgen;

3. die psychologische Abteilung, deren Aufgabe es ist, arbeitspsychologische und mit der Berufswahl verbundene Forschungs- und Unterrichtsarbeit sowie arbeitshygienische Fürsorgearbeit wenn nötig zusammen mit der medizinischen Abteilung auszuführen;

4. die physiologische Abteilung, deren Aufgabe es ist, die mit der Physiologie der Arbeit verbundene Forschungs- und Unterrichtsarbeit und Untersuchungen der Arbeitsfähigkeit auszuführen;

5. die technisch-hygienische Abteilung, deren Aufgabe es ist, zu dem Gebiet der Arbeitshygiene gehörende technisch-hygienische Forschungs- und Unterrichtsarbeit zu leisten.

Das Personal umfasste Anfang des Jahres 1951 60 Personen und wird bis zur Ende des Jahres noch ungefähr om 10—15 Personen wachsen.

Das Institut erhält staatliche Unterstützung, deren Grösse der Reichstag jährlich bestimmt. Diese Unterstützung soll im Jahre 1951 die Hälfte der Ausgaben des Instituts decken. Die andere Hälfte der Einkommen wird durch die eigene Tätigkeit des Instituts (c. 2/3) und durch Donationen (c. ⅓) erworben. Später wird die staatliche Unterstützung nur c. 30—35 % sein.